β-catenin is a component of stable cell adherent complexes whereas its free form functions as a transcription factor that regulate genes involved in oncogenesis and metastasis. Free β-catenin is eliminated by two adenomatous polyposis coli (APC)-dependent proteasomal degradation pathways regulated by glycogen synthase kinase 3β (GSK3β) or p53-inducible Siah-1. Dysregulation of β-catenin turnover consequent to mutations in critical genes of the APC-dependent pathways is implicated in cancers such as colorectal cancer. We have identified a novel retinoid X receptor (RXR)-mediated independent pathway in the regulation of β-catenin. In this proteasomal pathway, RXR agonists induce degradation of β-catenin and RXRα and repress β-catenin-mediated transcription. In vivo, β-catenin interacts with RXRα in the absence of ligand, but RXR agonists enhanced the interaction. RXR agonist action was not impaired by GSK3β inhibitors or deletion of the GSK3β-targeted sequence from β-catenin. APC and p53-mutated colorectal cancer cells, RXR agonists still inactivated endogenous β-catenin via RXRα. Interestingly, deletion of the RXRα A/B region abolished ligand-induced β-catenin degradation but not RXRα-mediated transactivation. RXRα-mediated inactivation of oncogenic β-catenin paralleled a reduction in cell proliferation. These results suggest a potential role for RXR and its agonists in the regulation of β-catenin turnover and related biological events.

β-catenin is a key mediator in Wnt regulation of multiple cellular functions in embryogenesis and tumorigenesis (1). In adult tissues, β-catenin is a component of stable cell adherent complexes whereas its free form functions as a co-activator for a family of transcription factors called T cell factor/lymphoid enhancer factor (TCF/LEF).1 Levels of free β-catenin are tightly regulated by two APC-dependent proteasomal degradation pathways, namely a GSK3β-regulated pathway involving the APC/Axin complex (2) and a p53-inducible pathway involving Siah-1 (3, 4). In the GSK3β-regulated pathway, β-catenin associates with the APC/Axin complex and undergoes a two-step phosphorylation by casein kinase I (CKI) and GSK3β at serine/threonine residues within the first 50 N-terminal amino acids (2, 5). β-catenin interacts with an ubiquitylation complex through the phosphorylated N terminus and undergoes proteasome-catalyzed degradation (6). Wnt inactivation of GSK3β leads to translocation of β-catenin to the nucleus, where it enables TCF/LEF to activate genes involved in embryogenesis and oncogenesis (1, 7). In the second pathway, p53-up-regulated Siah-1 interacts with the N-terminal region of APC, recruits an ubiquitylation complex to the N terminus of β-catenin, and targets it for proteasome-mediated degradation. Thus, both pathways require the intact N terminus of β-catenin. In cancers such as colorectal and hepatocellular cancers and melanoma, mutations in the key components of the two pathways, such as APC, p53, and Axin, or β-catenin itself, lead to dysregulation of β-catenin turnover and, consequently, high levels of nuclear β-catenin and abnormal activation of TCF/LEF-regulated genes that are involved in oncogenesis and metastasis (8, 9).

Retinoids, which are natural and synthetic derivatives of vitamin A, regulate gene transcription through two families of nuclear receptors, i.e. retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (10) and have significant anti-cancer effects (11, 12). These receptors are ligand-dependent DNA binding transcription factors. Each receptor has an N-terminal A/B region that harbors the ligand-independent activation function-1 (AF-1), a central DNA-binding domain (the C region), and a C-terminal E region containing a ligand binding domain and a ligand-dependent activation function-2 (AF-2). RARs and RXRs bind to target genes as RXR-RXR heterodimers or RXR homodimers. In the absence of ligands, retinoid receptors are associated with co-repressors and repress gene transcription (13). Once associated with agonists, RARs and RXRs undergo conformational changes, recruit co-activators, and activate target gene transcription. Interestingly, instances of crosstalk between the Wnt/β-catenin- and retinoid-signaling pathways have been reported recently. For example, RAR was found to interact with β-catenin in vitro and inhibits β-catenin-mediated gene transcription in vivo (14). Retinoic acid, an RAR agonist, was shown to synergize with Wnt signaling in the up-regulation of gene transcription (15, 16). Unlike the APC-dependent pathway, RAR signaling does not regulate β-catenin protein levels (14). On the other hand, RXR agonists have been shown to cause degradation of RXRs and also its receptor heterodimerization partners, including RARs and TR (17, 18). However, the biological consequences of such degradation phenomena have not been well understood. Here, we have investigated the role of RXR and its ligands in the

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1 The abbreviations used are: TCF/LEF, T cell factor/lymphoid enhancer factor (TCF/LEF).1 Levels of free β-catenin are tightly regulated by two APC-dependent proteasomal degradation pathways, namely a GSK3β-regulated pathway involving the APC/Axin complex (2) and a p53-inducible pathway involving Siah-1 (3, 4). In the GSK3β-regulated pathway, β-catenin interacts with an ubiquitylation complex through the phosphorylated N terminus and undergoes proteasome-catalyzed degradation (6). Wnt inactivation of GSK3β leads to translocation of β-catenin to the nucleus, where it enables TCF/LEF to activate genes involved in embryogenesis and oncogenesis (1, 7). In the second pathway, p53-up-regulated Siah-1 interacts with the N-terminal region of APC, recruits an ubiquitylation complex to the N terminus of β-catenin, and targets it for proteasome-mediated degradation. Thus, both pathways require the intact N terminus of β-catenin. In cancers such as colorectal and hepatocellular cancers and melanoma, mutations in the key components of the two pathways, such as APC, p53, and Axin, or β-catenin itself, lead to dysregulation of β-catenin turnover and, consequently, high levels of nuclear β-catenin and abnormal activation of TCF/LEF-regulated genes that are involved in oncogenesis and metastasis (8, 9).

Retinoids, which are natural and synthetic derivatives of vitamin A, regulate gene transcription through two families of nuclear receptors, i.e. retinoic acid receptors (RARs) and retinoic X receptors (RXRs) (10) and have significant anti-cancer effects (11, 12). These receptors are ligand-dependent DNA binding transcription factors. Each receptor has an N-terminal A/B region that harbors the ligand-independent activation function-1 (AF-1), a central DNA-binding domain (the C region), and a C-terminal E region containing a ligand binding domain and a ligand-dependent activation function-2 (AF-2). RARs and RXRs bind to target genes as RXR-RXR heterodimers or RXR homodimers. In the absence of ligands, retinoid receptors are associated with co-repressors and repress gene transcription (13). Once associated with agonists, RARs and RXRs undergo conformational changes, recruit co-activators, and activate target gene transcription. Interestingly, instances of crosstalk between the Wnt/β-catenin- and retinoid-signaling pathways have been reported recently. For example, RAR was found to interact with β-catenin in vitro and inhibits β-catenin-mediated gene transcription in vivo (14). Retinoic acid, an RAR agonist, was shown to synergize with Wnt signaling in the up-regulation of gene transcription (15, 16). Unlike the APC-dependent pathway, RAR signaling does not regulate β-catenin protein levels (14). On the other hand, RXR agonists have been shown to cause degradation of RXRs and also its receptor heterodimerization partners, including RARs and TR (17, 18). However, the biological consequences of such degradation phenomena have not been well understood. Here, we have investigated the role of RXR and its ligands in the
regulation of β-catenin activity and identified a novel RXR-mediated APC-independent pathway. We show that RXR agonists reduce β-catenin-mediated activation of gene transcription and cell proliferation through a protein degradation mechanism.

EXPERIMENTAL PROCEDURES

Retinoids—The RXR antagonist AGN194310 and the RXR agonist AGN194204 have been described previously (19, 20). The RXR-specific agonists AGN195362, AGN195456, AGN195741, AGN196060, and AGN196459 and the RXR-specific antagonist AGN195393 were synthesized at Allergan. Me$_2$SO was used as a solvent for the compounds.

Plasmids—TOPFLASH, which contains TCF/LEF binding sites placed in front of the TK-Luc reporter gene, was purchased from Upstate Biotechnology. The β-catenin expression vector, Gene Storm clone H-X87838 M in pcDNA3.1/RS, was purchased from Invitrogen. Nβ-catenin, a β-catenin mutant with an N-terminal deletion (amino acid residues 1–50), was made by PCR amplification from wild type β-catenin using the following pair of primers: 5′-AGG CAT TCA ACC AGG AAT CCT GAG GAA GAG-3′ and 5′-AGT CTA GAC CAT AGG TCA GTA TCA AAC GAG-3′. The resulting fragment was cloned into expression vector pCDNA3.1+ (Invitrogen Corp) between BamH1 and XhoI and confirmed by DNA sequencing. Finally, the N-terminal coding region was changed every 3 days, and individual zeocin-resistant clones were isolated. Clones stably expressing β-catenin or ∆Nβ-catenin were identified by Western blotting.

To produce CATXα and mCATXα cell lines that stably express RXRs with wild type β-catenin or mutant ∆Nβ-catenin, pGS-β-catenin and pGS-∆Nβ-catenin were transfected into cell line Xα that stably expresses FLAG-tagged RXRα. These cell lines were established as described above.

Proteasome inhibitors—Proteasome inhibitors MG132 and MG262 were purchased from Biomol Research Laboratories and Calbiochem, respectively. Lysosomes inhibitors, bafilomycin, leupetin, E-64, and ammonium chloride were purchased from Sigma-Aldrich.

Reporter Gene Assays—For measuring the TOPFLASH activity, cells were seeded at 50,000 cells per well in 24-well plates coated with poly-D-lysine. Twenty-four hours later, TOPFLASH and expression vectors were co-transfected into cells using FuGENE (Roche Applied Science) in DMEM containing 10% charcoal-treated FBS. To monitor transfection efficiency, either 15 ng of pHRG-TK renilla or 100 ng of CMX-LacZ DNA were co-transfected. Five hours later, vehicle or retinoids were added. The cells were treated for 16 h before harvest. Luciferase activity was measured using the Dual luciferase reporter 100 assay system (Promega). Control Renilla activity was determined using the same kit. A second control, β-galactosidase activity, was measured by colorimetric assays. The reporter activity was normalized against either β-galactosidase or Renilla activity.

Analysis of RXRs and its mutants in transactivation was performed as described above. To test for RXRs and RXRα in 3.5 × 10$^5$ CV-1/3A/LCS.

Human RXRα cDNA in a human keratinocyte cDNA library (21) was identified in a yeast two-hybrid system using RARy as a bait. The RXRα coding region was amplified from this clone by PCR using a pair of following primers: 5′-AGG GAA TTC ATG ACC GAC AAA CAT TTC CTG CCG-3′ and 5′-AGG CTG CAG CAT AGT TTG GTG CGG CTC-3′. The resulting fragment was subcloned into pEGFP-N2 (Clontech) between the EcoRI and PstI sites and then released by EcoRI and KpnI digestion. The released RXRα coding region was then cloned into a modified pCMV-FLAG vector (Sigma) containing the FLAG epitope DYKDDDDK. The RXRα deletion mutants (see Fig. 6A) were constructed by PCR amplification of hRXRα DNA using primers as follows: 5′-AGG ATC TCT GCC CGA TCG ACC GCC GC-3′ and 5′-AGG ACC CCC TCA GTC TCG AGG ACC GC-3′; 5′-AGG GAT CCC TAA GTC ATT TGG TGC GGC GCC TCC-3′ for RXRαCDE; 5′-AGG AAT TCA AGC GAG AGG TCG CAG AGG AGG AGG GC-3′ and 5′-AGG GAT CCC TAA GTC ATT TGG TGC GGC GCC TCC-3′ for RXRαDE; 5′-AGG AAT TCT CCC CCA GAG ACC TCT TCA CAA GC-3′ and 5′-AGG GAT CCC TAA GTC ATT TGG TGC GGC GCC TCC-3′ for RXRαED; 5′-AGG AAT TCT CCC CCA GAG ACC TCT TCA CAA GC-3′ and 5′-AGG GAT CCC TAA GTC ATT TGG TGC GGC GCC TCC-3′ for RXRαE; and 5′-AGG AAT TCA TGG ACA ACC ATC ATT TCC CGG-3′ and 5′-AGG GAT TCT ATG TGC TGG AGG AAG AGG AGG GC-3′ for RXRαΔAF2. The resulting PCR fragments were cut by EcoRI and KpnI and cloned into the pCMV-FLAG vector. For construction of RXRαC and RXRαCΔCD, the EcoRI fragment containing the A/B region of RXRα was obtained by PCR amplification with primers 5′-AGG AAT TCA TGG ACA ACC ATC ATT TCC CGG-3′ and 5′-AGG AAT TCA TGG ACA ACC ATC ATT TCC CGG-3′ and inserted into constructions RXRαDE and RXRαE at the EcoRI site in front of the DE and E regions of RXRα, respectively. For making RXRαΔCD, the EcoRI fragment containing the ABC region of RXRα was prepared by PCR amplification using the following primers: 5′-AGG AAT TCA TGG ACA ACC ATC ATT TCC CGG-3′ and 5′-AGG AAT TCA TGG ACA ACC ATC ATT TCC CGG-3′ and inserted into the EcoRI site in front of the E region in construct RXRαE.

Antibodies—Native or horseradish peroxidase (HRP)-conjugated mouse monoclonal antibodies against the FLAG tag in RXRα, M2, and HRP-M2 were purchased from Sigma. Native or HRP-conjugated mouse monoclonal antibodies against the V5 tag in β-catenin and HRP-V5, respectively, were purchased from Invitrogen. Rabbit polyclonal antibodies against the N-terminus of RXRα (D20), the C terminus of β-catenin (H102), poly(ADP-ribose) polymerase (PARP, H-250), GSKβ (H76), and a mouse monoclonal antibody against β-tubulin (D-10) were purchased from Santa Cruz Biotechnology, HeLa, CV-1, and SW480 cells were purchased from the American Type Culture Collection and grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 10 μg/ml streptomycin at 37 °C in 5% CO$_2$. To generate the cell lines CAT and mCAT that stably express RXRs, 1×10$^6$ cells were transfected into HEK293 cells using LipofectAMINE. Twenty-four hours later, the cells were subjected to selection in the presence of zeocin (Invitrogen) at 400–500 μg/ml. The selection medium was changed every 3 days, and individual zeocin-resistant clones were

In Vivo Protein Crosslinking and Immunoprecipitation—HEK293 cells at ~80% confluence in 150-mm poly-D-lysine-coated plates (BD Biosciences) were transfected with expression vectors for β-catenin (8 μg), ∆Nβ-catenin (8 μg), and RXRα (4 μg) and cultured overnight in DMEM containing high glucose and 10% activated charcoal-treated FBS. To monitor transfection efficiency, either 15 ng of pHRG-TK renilla or 100 ng of CMX-LacZ DNA were co-transfected. Five hours later, vehicle or retinoids were added. The cells were treated for 16 h before harvest. Luciferase activity was measured using the Dual luciferase reporter 100 assay system (Promega). Control Renilla activity was determined using the same kit. A second control, β-galactosidase activity, was measured by colorimetric assays. The reporter activity was normalized against either β-galactosidase or Renilla activity.

Analysis of RXRs and its mutants in transactivation was performed as described above. To test for RXRs and RXRα in 3.5 × 10$^5$ CV-1/3A/LCS.
were lysed in ice-chilled radioimmuno precipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in phosphate-buffered saline) containing a mixture of protease inhibitors (Sigma) and homogenized by QIAshredder (Qiagen). The extracts (1.5 mg per immunoprecipitation) were incubated overnight with specific antibodies and protein G-agarose beads with constant shaking at 4 °C. Immunoprecipitated materials were washed with the ice-cold radioimmuno precipitation assay buffer and dissolved in SDS-PAGE loading dye containing β-mercaptoethanol by heating at 100 °C for 5 min. This procedure frees the DSP-crosslinked molecules pulled down by the antibodies. Proteins were resolved on 4–12% SDS-polyacrylamide gels followed by Western blotting.

Cell Proliferation Assay—Cell proliferation assays were performed in 96-well microtiter plates. HEK293, mCAT (HEK293-derived cells stably expressing ΔNβ-catenin), Xα (HEK293-derived cells stably expressing RXRα), and mCATXα cells were seeded at 200–400 cells/well in regular growth medium. The next day, vehicle or retinoids were added. Cell proliferation was measured after 6 days of treatment using a cell proliferation kit purchased from Chemicon International.

**RESULTS**

**RXR Agonists Inactivate β-Catenin-mediated Transcription via Endogenous and Transfected RXRs**—We investigated the effect of RXR-specific agonists on β-catenin-mediated TCF/LEF transcriptional activity, a surrogate marker for the oncogenic activity of β-catenin, using the TOPFLASH reporter gene, which contains TCF/LEF binding sites (22, 23). In HEK293 cells, the significant reporter activity produced by endogenous β-catenin was reduced by AGN194204, an RXR-specific agonist, in the absence (~50%) or presence (~70%) of transfected RXRα (Fig. 1, a and b). The significantly increased reporter activity obtained with β-catenin transfection was still very effectively (~80%) reduced by AGN194204 treatment in the presence of cotransfected RXRα (Fig. 1, a and b). Similar inhibition was observed in CATXα cells that were stably transfected with both β-catenin and RXRα, whereas the AGN194204 effect was less pronounced in CAT cells that were stably transfected with only β-catenin (Fig. 1c).

**RXR Agonists Induce β-Catenin Degradation via Endogenous and Overexpressed RXRs**—Because APC-dependent protein degradation pathways regulate β-catenin-mediated transcription (6, 22, 23) and RXR agonists induce degradation of
RXR and associated receptor partners (17, 18), we sought to determine whether AGN194204 inhibited TOPFLASH reporter activity by reducing β-catenin protein levels. HEK293 cells were treated with vehicle or the RXR agonist, and total cell lysates were analyzed by immunoprecipitation and Western blotting. As shown in Fig. 2a, AGN194204 decreased endogenous β-catenin (−50%) and RXRα levels in HEK293 cells in the absence of transected RXRα. AGN194204 further reduced endogenous β-catenin (−80%) in the presence of transected RXRα. In HEK293 cells transfected with β-catenin alone, AGN194204 had no effect on β-catenin because of the low levels of endogenous RXRα relative to transected β-catenin (Fig. 2b).
However, it dramatically reduced β-catenin protein levels concurrent with RXRα protein levels in cells transfected with both RXRα and β-catenin. Similar AGN194204 effects were obtained in stably transfected HEK293 cells (CATXα; Fig. 2c) or transiently transfected CV1, HeLa, and SW480 cells (Fig. 2e), indicating the ubiquitous nature of this phenomenon. The AGN194204 effects on reducing β-catenin protein levels were time- (Fig. 2e) and dose-dependent (Fig. 2f), and the efficiency of the reduction depended on RXR protein levels (Fig. 2, a and d). AGN194204 readily caused a significant decrease of β-catenin at a dose as low as 1 μM (Fig. 2f), reflecting its high affinity for RXRα. Several different RXR agonists, including AGN195362, AGN195456, AGN195741, AGN196060, AGN196459, and 9-cis retinoic acid, similarly reduced β-catenin protein levels (Fig. 2g, and data not shown). An RXR-specific antagonist, AGN195393 (24), dose-dependently inhibited the AGN194204 effects on β-catenin and RXR protein levels (Fig. 2f). An RXR agonist, TTNPB, or an RXR antagonist, AGN194310, showed no effects (Fig. 2g). Pulse-chase analysis was performed to ascertain whether the RXR agonist effect occurs at the level of protein degradation. AGN194204 accelerated degradation of both 35S-labeled RXRα and β-catenin (Fig. 2h). In the presence of cycloheximide and the absence of AGN194204, β-catenin is readily subjected to degradation by the active APC-pathways in HEK293 cells (compare lane 3 to lane 1 in Fig. 2i) as expected. However, cycloheximide did not block the AGN194204-induced degradation of β-catenin and RXRα (comparing lane 4 to lane 3 in Fig. 2i), indicating that induction of transcriptional activity is not required for this effect. Together, these data indicate that RXR agonists reduce β-catenin protein levels by an RXR-mediated protein degradation pathway, which is independent of the RXR-mediated gene transcription activation pathway.

In cells where the Siah- and GSK3β-regulated APC pathways are impaired by mutations or GSK3β is inhibited by Wnt signaling, levels of β-catenin are increased in the cytoplasmic compartment, and, ultimately, β-catenin is translocated to the nucleus where it transactivates the TCF/LEF-targeted genes. Our transactivation data indicated that nuclear β-catenin-related transcriptional activity was reduced by RXR agonists. We further examined whether inhibition of β-catenin-mediated gene transcription by AGN194204 is due to a reduction of β-catenin protein levels in the nucleus with an analysis of nuclear and cytosolic fractions of CATXα cells. Poly(ADP-ribose) polymerase and β-tubulin were used as nuclear and cytoplasmic markers, respectively, for monitoring the efficiency of separation of the two fractions. Decreases in β-catenin protein levels as a result of AGN194204 treatment were observed in both nuclear and cytosolic compartments (Fig. 2, j and k).

The APC-dependent degradation of β-catenin and the agonist-dependent degradation of RXR proceed by proteasomal pathways (6, 17, 18). To determine whether the RXR agonist-induced degradation of β-catenin involves this pathway, we treated cells with the proteasome inhibitors MG262 and MG132. As shown in Fig. 2, l and m, these two inhibitors dose-dependently blocked AGN194204-induced degradation of both β-catenin and RXRα. However, lysosomal inhibitors such as bafilomycin, E64, NH4Cl, and leupeptin had no effect (data not shown). These data indicate that RXR agonist-induced degradation of β-catenin also proceeds by a proteasomal pathway.

The RXR-regulated β-Catenin Degradation Pathway Is Independent of the p53/Siah-1 and GSK3β-regulated APC Pathways—To determine whether the GSK3β- or p53/Siah-regulated APC pathways are involved in the RXR agonist effects, a β-catenin mutant (ΔNβ-catenin) with a deletion of the N-terminal sequence (50 amino acids) that is targeted by the two APC-dependent pathways was prepared (6, 23). Although this mutant, which is resistant to APC-mediated degradation pathways, showed higher TOPFLASH reporter gene activity than wild-type β-catenin, its increased activity was very effectively inhibited by AGN194204 in the presence of RXRα (Fig. 1d).

High levels of TOPFLASH reporter activity associated with elevated β-catenin levels have been reported in SW480 colorectal cancer cells wherein both APC and p53 genes contain loss-of-function mutations (22, 23, 25). However, AGN194204 effectively inhibited reporter activity in SW480 cells containing cotransfected RXRα (Fig. 1e), which is consistent with the observed decrease in endogenous β-catenin levels in these cells (Fig. 2a). Similarly, whereas LiCl, a GSK3β inhibitor that is known to elevate free β-catenin levels (26) significantly increased TOPFLASH reporter activity, AGN194204 still effectively reduced this elevated activity (Fig. 1f). At the protein level, AGN194204 induced degradation of ΔNβ-catenin as effectively as that of wild-type β-catenin in transiently (Fig. 2e) or stably transfected cells (Fig. 2c). Together, these data clearly indicate that the RXR-mediated degradation of β-catenin does not involve the APC-dependent pathways.

β-Catenin Interacts with RXRα in Vivo—Our data showed that RXR agonist-induced new protein synthesis was not required for β-catenin degradation (Fig. 2i), whereas RXR protein degradation appeared to be essential for the process (Fig. 2d and Ref. 21). These observations suggested that RXRα and β-catenin proteins interacted directly or were present in the same degradation complex. To test this hypothesis, HEK293 cells were transfected with a combination of expression vectors for RXRα and wild type β-catenin or ΔNβ-catenin. The cells were treated with AGN194204 and subjected to cross-linking
using the reversible cross-linker DSP prior to cell lysis and immunoprecipitation. Both β-catenin and ΔNβ-catenin were pulled down by the FLAG antibody only in cells cotransfected with FLAG-RXRα (Fig. 3a), and, conversely, RXRα was pulled down by V5 antibody only in cells cotransfected with V5-tagged β-catenin proteins (Fig. 3b). Under the same conditions, endogenous GSK3β was co-immunoprecipitated with β-catenin but not with RXR, thereby indicating that the RXRα/β-catenin interaction is specific and does not involve GSK3β. Although AGN194204 enhanced the effect, RXRα interacted with β-catenin even in the absence of an RXR agonist.

**RXR Agonist-induced β-Catenin Degradation and Transactivation Are Two Separable Functions of RXRα**—To determine the functional domains of RXRα associated with degradation of β-catenin, various deletions were introduced into the receptor (Fig. 4a). Helix 12 (AF-2) of RXRα was required for both self and β-catenin degradation as would be expected for agonist-induced functions (Fig. 4b). RXRα mutants carrying C and/or D region deletions were able to mediate agonist-induced degradation of β-catenin, albeit with reduced efficiency relative to the wild-type receptor. A/B region deletion mutants were particularly ineffective in mediating β-catenin degradation, although the mutant receptors themselves underwent degradation in response to AGN194204. Interestingly, RXRα CDE, an A/B region deletion mutant, was fully effective in agonist-induced gene transcription (Fig. 4c), indicating that β-catenin
degradation and transcriptional activation are two separable functions of RXRa.

**RXR Agonists Inhibit Growth of ΔNβ-Catenin-expressing Cells via RXR—Dysregulation of β-catenin by mutations in the N-terminal GSK3β-targeted sites is associated with cancer cell growth. Specific reduction of β-catenin protein levels by antisense oligonucleotides or small interference RNA in APC mutant colon cancer cells inhibited cell proliferation, anchorage-independent growth, and cellular invasiveness in vitro (27, 28).** We have investigated whether the RXRa-mediated degradation of ΔNβ-catenin affected the cell proliferation rate. The growth of mCATα cells, which stably express both ΔNβ-catenin (mCAT) and RXRa, was effectively inhibited by AGN194204 in a dose-dependent manner, whereas HEK293-cells that stably express either ΔNβ-catenin (mCAT) or RXRa (Xα) alone are not substantially impaired by AGN194204 treatment (Fig. 5). The IC50 for the growth inhibitory effect of AGN194204 was ~1 nm, which was consistent with the affinity of this ligand for RXRa and also its potency in inducing β-catenin degradation (Fig. 2f).

**FIG. 5.** RXR agonists inhibit growth of ΔNβ-catenin-expressing cells via RXRa. The stable cell lines mCAT, Xα, and mCATXα and their parental cell line HEK293 were treated with vehicle or AGN194204 for 6 days. Cell proliferation rate was measured using a cell proliferation kit (Chemicon). Data are mean ± S.E. from more than six samples.

**Fig. 6.** A schematic model of three pathways in regulation of β-catenin turnover. Pathway-specific components are listed. β, frizzled; dsh, dishevelled.

**FIG. 6.** A schematic model of three pathways in regulation of β-catenin turnover. Pathway-specific components are listed. β, frizzled; dsh, dishevelled.

**Mechanism by Which RXR Agonists Induce Degradation of β-Catenin—**Our data clearly indicated that stoichiometric levels of RXRa were required for the efficient degradation of β-catenin (Fig. 2, a and d), indicating that RXRa and β-catenin are very likely present in the same degradation complex. This was confirmed by our observation that β-catenin interacted with RXRa in intact cells (Fig. 3). The interaction appears to be transient, because we were not able to detect it under conventional immunoprecipitation conditions and also because GST-β-catenin had previously been shown not to interact with in vitro translated RXRa (14). Examination of the β-catenin protein sequence revealed the presence of five consensus LXXLL receptor-interacting motifs, which are usually found in co-activators and co-repressors that interact with nuclear receptors. Whether these serve as RXR-interacting motifs in β-catenin remains to be determined.

**RXR agonists** have been reported previously to cause RXR-mediated degradation of RXR dimerization partners such as RAR and TR. The degradation of these receptor heterodimers is achieved by the ubiquitin-mediated proteasomal system (17, 18). Heterodimeric partners of RXRs such as RARs and TR have been found to be co-degraded with RXRs in the presence of RXR agonists (17, 18). When each member of the RAR family was co-transfected with β-catenin, treatment with RXR agonist TTDNB or RXR agonist AGN194204 did not significantly alter β-catenin protein level (data not shown). However, when RXRa was included in the co-transfection, the RXR agonist induced degradation of β-catenin efficiently (data not shown). Under the same condition, all three members of the RAR family and RXRa were degraded when AGN194204 was added. These observations suggest that the RXR agonist induced β-catenin degradation via RXR and that heterodimeric partners such as RARs are not required in the regulation of β-catenin by RXR agonists. We showed that proteasome inhibitors blocked RXR agonist-induced degradation of both β-catenin and RXR, indicating that the process involves a proteasomal pathway (Fig. 2, l and m). Thus, it appears that a broad spectrum of RXR-interacting proteins, exemplified by RAR, TR and β-catenin, can be targeted for degradation by RXR agonists. It should be noted that RXR interacts with these various proteins in the absence of a ligand, although the interaction may be enhanced by agonists. However, dimerization partners were not required for RXR degradation by RXR agonists (17, 18), and our results are consistent with these observations. Upon ligand binding, RXRa itself changes conformation, is subjected to modification, and becomes a target of the ubiquitin-proteasome machinery (17, 18). Two distinct mechanisms can be envisaged for the
degradation of RXR-targeted proteins. In the first scenario, RXRα serves as a docking and regulatory protein for its targets, which undergo parallel changes in response to RXR agonists and become substrates of the proteasome machinery. In the second scenario, RXRα serves as a targeting molecule that carries interacting proteins to certain cellular compartments for modification and degradation. Given the complexity of the APC-dependent β-catenin regulatory machinery, the RXRα-mediated pathway may also similarly involve multi-protein complexes and multi-step reactions. Further studies will be required to elucidate the details of this intriguing pathway.

Two Separate Functions of RXRα Are β-Catenin Protein Degradation and Transcriptional Activation—In this study, we have compared the effects of deletion of different RXR functional domains on the degradation of β-catenin and RXRα and on RXR-mediated transactivation (Fig. 4). This comparison revealed several key differences. First, the A/B region of RXRα was required for agonist-induced β-catenin degradation but not for transactivation. Second, the integrity of RXRα was required for efficient degradation of β-catenin, whereas degradation of RXRα itself was less sensitive to the loss of certain functional regions as illustrated by the sensitivity of RXRαE, which contains only the ligand-binding domain, to AGN194204-induced degradation. In addition, our experiment using cycloheximide indicated that the RXR agonist effects on β-catenin and RXRα degradation do not require de novo protein synthesis (Fig. 2c).

In other words, transactivation of RXR-regulated genes was not required for β-catenin degradation. These observations suggest that the requirements for β-catenin degradation are different than those for transactivation, indicating that ligand-mediated protein degradation and transcriptional activation are two separable functions. In support of this view, Osburn et al. have shown that ligand-induced degradation of RXRα is independent of its transcriptional activity and does not require interaction with a co-activator. On the other hand, helix 12 (AF-2) of RXRα is essential for all agonist-mediated biological activities. This is not unexpected, because repositioning of helix 12 is largely responsible for the changes in RXRα conformation caused by agonist binding (29). Although conformational changes of this type are necessary for both β-catenin degradation and transactivation functions, it is possible that the optimal conformation of RXRα for inducing β-catenin degradation may differ from that for transactivation. In summary, our results have identified a novel ligand-dependent function for RXRα, namely that of targeting oncogenic proteins for degradation, which is distinct from its role in the regulation of gene transcription.

β-Catenin and Cell Growth—Dysregulation of β-catenin turnover by mutations in the N-terminal GSK3β-targeted sites is associated with cancer cell growth. For example, expression of β-catenin with gain-of-function mutations such as mutations or deletions of its N-terminal casein kinase I/γ glycogen synthase kinase 3β phosphorylation sites caused tissue neoplastic growth in animals (30–35). Overexpression of similar β-catenin mutants leads to neoplastic transformation of E1A-immortalized epithelial cells and stimulated proliferation of p53- or ARF-null mouse embryo fibroblasts (36, 37). Consistent with these results, specific reduction of β-catenin in APC-mutant colon cancer cells by antisense oligonucleotides or small interference RNA inhibited the proliferation, anchorage-independent growth, and cellular invasiveness in vitro and neoplastic growth of xenografts in animals (27, 28, 38). In keeping with the role of β-catenin in cell growth, activation of the RXRα-mediated pathway reduced cell proliferation (Fig. 5) in parallel with the reduction in ΔNβ-catenin protein levels and transcriptional activity (Figs. 1 and 2).

The Potential Role of the RXRα Pathway in Cancer Therapy—There are four major pathological events that cause a slow turnover of β-catenin in cancer as follows: 1) mutations of the Axin- and β-catenin-interacting motifs in APC; 2) mutations of the N-terminal GSK3β phosphorylation sites in β-catenin; 3) mutations in Axin; and 4) inactivation of GSK3β by the Wnt signaling pathway (39). These events are believed to be the major factors underlying pathogenesis of cancers such as colorectal cancer and melanoma. Mechanistically, these events lead to the escape of β-catenin from cellular surveillance and subsequently to the pathogenic activation of genes involved in tumorigenesis and metastasis. Thus, in cancers involving dysregulation of β-catenin turnover, the following two therapeutic approaches could be considered: 1) restoration of the regulatory machinery; or 2) activation of a pharmacological pathway to reduce free β-catenin. The first approach is not practical, because the high frequency of mutations in key genes constituting the APC pathways, such as APC, Axin, p53, and β-catenin itself are the origin of the problem (Fig. 6). In the second approach, manipulation of the p53 and the Wnt signaling pathways have not been successful for various reasons, including the aforementioned genetic problems. However, the RXR-mediated pathway, which can be regulated by small molecule hormones, has the potential of being a powerful pharmacological approach to treating Wnt/β-catenin-related cancers. Our results suggest that β-catenin-associated tumors that currently express high levels of RXR will be the most responsive to RXR agonist therapy. Furthermore, the use of RXR agonists in conjunction with pharmacological (40) or genetic (41) approaches to elevating RXRα protein levels in target tumors may be effective therapies for cancers such as colorectal cancer. We are currently investigating these possibilities using in vivo model systems.

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