Protection against Anoikis and Down-regulation of Cadherin Expression by a Regulatable β-Catenin Protein*

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β-Catenin signaling plays a key role in a variety of cellular contexts during embryonic development and tissue differentiation. Aberrant β-catenin signaling has also been implicated in promoting human colorectal carcinomas as well as a variety of other cancers. To study the molecular and cellular biological functions of β-catenin in a controlled fashion, we created a regulatable form of activated β-catenin by fusion to a modified estrogen receptor (ER) ligand binding domain (G525R). Transfection of tissue culture cells with expression vectors encoding this hybrid protein allows the signal transduction function of β-catenin to be induced by the synthetic estrogen, 4-hydroxytamoxifen, leading to regulated activation of a β-catenin-lymphocyte enhancer-binding factor-dependent reporter gene as well as induction of endogenous cyclin D1 expression. The activation of ER-β-catenin signaling rescues RK3E cells from anoikis and correlates with an increased phosphorylation of mitogen-activated protein kinase. The inhibition of anoikis by ER-β-catenin can be abolished by a mitogen-activated protein kinase pathway inhibitor, PD98059. Evidence is also provided to show that ER-β-catenin down-regulates cadherin protein levels. These findings support a key role for activated β-catenin signaling in processes that contribute to tumor formation and progression.

Wnt-1 was first identified as a proto-oncogene in mouse mammary tumors (1). Wnt-1 and other members of this gene family normally play important roles in embryonic development including the specification of cell fate, induction of body axis, and determination of embryonic patterning (2–4). The Wnt genes encode secreted glycoproteins that function as ligands for Frizzled family seven-transmembrane receptors (3, 5–11) and its coreceptors Dally and LRP (12–16). Binding of some Wnt proteins to their partner Frizzled receptors activates β-catenin-mediated signal transduction. The cytoplasmic constituents of this pathway include the disheveled protein that is recruited to the plasma membrane upon binding of Wnt to Frizzled and leads to inhibition of the activity of glycogen synthase kinase 3β, a negative regulator of Wnt/β-catenin signaling (17, 18). Glycogen synthase kinase 3β is found in a complex with the tumor suppressor protein, adenomatous polyposis coli (APC)* and axin/conductin (19–22). When active, glycogen synthase kinase 3β phosphorylates β-catenin at its N terminus and facilitates the rapid degradation of β-catenin by a ubiquitin-proteosome pathway (21, 23–27).

β-Catenin was initially described as a protein that interacts with the cytoplasmic tail of the transmembrane, cell-to-cell adhesion molecule, E-cadherin (28, 29) and couples E-cadherin to the actin cytoskeleton via a-catenin (30–32). The association with β-catenin is necessary for cadherin-mediated cell-to-cell adhesion, and β-catenin also serves a non-cadherin-dependent signal transduction function in a variety of cellular contexts (33–35). Activation of the β-catenin pathway by Wnt-1 leads to the accumulation of a cytoplasmic pool of β-catenin (36, 37), which then translocates into the nucleus and binds to transcription factors of the lymphocyte enhancer-binding factor 1 (LEF-1/T cell family to regulate β-catenin-LEF-dependent gene expression (3, 38–41). In addition to the Wnt proteins, other growth factors such as epidermal growth factor (42) and hepatocyte growth factor can lead to elevated cytoplasmic β-catenin and enhanced β-catenin-LEF-dependent transcription (43). Another positive regulator of the β-catenin pathway, integrin-linked kinase, has also been found to promote nuclear translocation of β-catenin and transcriptional activation by the β-catenin-LEF complex (44).

Deregulated β-catenin signaling has been observed in human tumor cells and is thought to play a pivotal role in the genesis of a variety of malignancies (33–35, 45). In particular, functionally inactivating mutations of the APC gene have been detected in more than 80% of colorectal cancers (46–50). Mutational inactivation of the APC protein contributes to the accumulation of β-catenin and deregulated expression of its downstream target genes (such as c-myc, WISP, and cyclin D1), some of which have also been implicated in human cancers (51–55). In many tumors expressing wild-type APC, constitutive activation of the β-catenin-LEF signaling pathway can be attributed to mutations of the N-terminal phosphorylation sites in β-catenin (Ser23, Ser37, Thr41, and Ser45), which result in stabilization of the protein and consequent signal activation (56–58).

Although the pivotal role of β-catenin in malignant transformation is well substantiated, the cell biological consequences of β-catenin signaling are not clearly defined. To study the func-

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1 The abbreviations used are: APC, adenomatous polyposis coli; ER, estrogen receptor; FACS, fluorescence-activated cell sorter; HA, hemagglutinin; HBD, hormone binding domain; 4-HT, 4-hydroxytamoxifen; LEF-1, lymphocyte enhancer-binding factor 1; MAP, mitogen-activated protein; poly-HEM, poly-(2-hydroxyethyl methacrylate).
tions of β-catenin in a controlled fashion, we created a regulatable form of activated β-catenin by fusion of the entire protein (β-catenin S37A/A45A) to a modified estrogen receptor (ER) ligand binding domain (G525R) (59). Expression of this protein in several cell lines by transfection allows the signal transduction function of β-catenin to be induced by 4-hydroxytamoxifen (4-HT), leading to activation of a β-catenin-LEF-dependent reporter construct. Using this inducible system we demonstrate that β-catenin signaling correlates with diminished cell-substrate adhesion and can rescue RK3E cells from anoikis by a process that appears to involve MAP kinase activation. We also provide evidence that ER-β catenin down-regulates cadherin protein levels. These findings support a key role for enhanced β-catenin signaling in processes that contribute to tumor formation and progression.

EXPERIMENTAL PROCEDURES

Vectors—Human β-catenin was first amplified from a cDNA library by PCR, and site-directed mutagenesis was then used to generate the activated mutant form of β-catenin (S37A/A45A). Restriction sites were engineered into PCR primers which allowed fusion of the modified hormone binding domain of the murine estrogen receptor (ER-HBD) in-frame with β-catenin S37A/A45A. To generate ER-β-catenin, ER-HBD G525R was amplified by PCR and cloned into an in-house mammalian expression vector under the control of the human cytomegalovirus promoter following five copies of LEF binding sites. For the control vector, plasmids were sequenced to ensure that no mutations had been introduced into the constructs during PCR amplification. Both chimeras were expressed with an N-terminal epitope tag encoding a 16-amino acid portion of the Haemophilus influenzae hemagglutinin (HA) gene. A β-catenin-LEF-responsive reporter gene was constructed by linking the coding region of firefly luciferase to the 3′-untranslated region of the chimerical promoter following five copies of LEF binding sites. For the vector control, Renilla luciferase was used to normalize the transfections and reporter assays. The Renilla luciferase gene is under the control of a constitutive thymidine kinase promoter (Promega, Madison, WI).

Cells and Transfections—293T cells were obtained from GenHunter Corporation (Nashville, TN), and RK3E cells were purchased from American Type Culture Collection. These were cultured in modified Eagle’s medium (with 4.5 g/liter glucose) supplemented with 10% fetal bovine serum. 105 cells were seeded into each well of a 12-well plate and transfected overnight and then cultured in standard growth medium with or without 1 μM 4-HT for 2 days. The cells were then fixed with ice-cold methanol-acetone (1:1) at −20 °C for 10 min. After incubation in blocking buffer (1% bovine serum albumin in phosphate-buffered saline) for 10 min, the cells were incubated with antisera to β-catenin-expressing RK3E cells (clone 27) were grown with or without 1 μM 4-HT (Sigma) for 2 days. Cell extracts were prepared using Nonidet P-40 lysis buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris-HCl, pH 7.4). Cells were then harvested, washed, and stained with annexin V-fluorescein isothiocyanate antibodies (PharMingen) and analyzed by flow cytometry using FACScalibur (Becton Dickinson). Because the pool of positive control cells infected with the β-catenin S37A/A45A-expressing retrovirus contained both expressing and nonexpressing cells, the percentage of viable RK3E cells that expressed β-catenin S37A/A45A (green fluorescence protein–positive population) was compared with the

Immunoprecipitation and Western Blot Analysis—RK3E cells expressing ER-β-catenin (clones 27 and 96) and a control clone with no expression of ER-β-catenin were grown with or without 1 μM 4-HT for 2 days. Cell extracts were prepared using RIPA lysis buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris-HCl, pH 7.4), incubated on ice for 30 min (RIPA lysis buffer–supplemented protease inhibitor mixture tablets (Roche).) Following centrifugation at 15,000 × g for 15 min, the supernatants were collected and concentrated for Western blot analysis. The pellet of each sample was then washed three times with Nonidet P-40 lysis buffer and solubilized in RIPA buffer and analyzed as the Nonidet P-40-insoluble fraction. Approximately 250 μg of the Nonidet P-40-soluble fraction was precipitated with 5 μg of anti-E-cadherin (Transduction Laboratories) or anti-catenin (Santa Cruz Biotechnology) antibodies in a total volume of 1.2 ml. After incubation at 4 °C for 2 h, 50 μl of agarose beads (Aversham Biotechnology) was added to each sample. After 1-h incubation at 4 °C, the beads were washed five times with Nonidet P-40 lysis buffer plus 1% Triton X-100 (Sigma). Western blot analysis was performed as described above.

Immunofluorescence Staining—An RK3E cell line expressing ER-β-catenin (clone 27) and a control RK3E cell line (clone 152) with no expression of ER-β-catenin were first grown in eight-well Lab-Tek II chamberslides (Nalgen Nunc International) on coverslips to allow cell attachment overnight and then cultured in standard growth medium with or without 1 μM 4-HT for either 1 or 2 days. The cells were then fixed with ice-cold methanol-acetone (1:1) at −20 °C for 10 min. After incubation in blocking buffer (1% bovine serum albumin in phosphate-buffered saline) for 10 min, the cells were incubated with antisera to β-catenin (1:500, Transduction Laboratories), E-cadherin (1:50, Transduction Laboratories) and HA tag (Corporation) or anti-mouse antibodies (Jackson Immune Research) at a dilution of 1:200 for 45 min at room temperature. After brief washing, the localization of β-catenin was visualized by fluorescence microscopy.

RNA Expression Analysis—Control RK3E cells (clone 4) or ER-β-catenin-expressing RK3E cells (clone 27) were grown with or without 4-HT treatment for 1 or 2 days. A total of 1.2 ml of each cell lysate sample was transferred to a 1.5-ml microcentrifuge tube (Falcon) and contaminating genomic DNA was removed by DNase I treatment. The expression of cyclin D1, a gene known to be transcribed following 5 μg of DNA using Effectene (Qiagen) according to the manufacturer’s recommendation. For generation of stable transfected cell lines, RK3E cells were transfected with the expression plasmid encoding ER-β-catenin along with a selection plasmid pDNA3.1 (−) (Invitrogen) encoding a neomycin resistance gene at a ratio of either 10:1 or 50:1 (ER-β-catenin:pDNA3.1). Two days later, the cells were split at a 1:10 dilution, and the following day, the standard culture medium was replaced with standard medium containing either 300 or 800 μg/ml Geneticin (Invitrogen). The selective medium was changed every 3–4 days, and 2 weeks later 60 clones were picked and expanded. To select for clones expressing ER-β-catenin, the clonal lines were transfected individually with the β-catenin-LEF reporter plasmid, cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum in the presence or absence of 1 μM 4-HT (Sigma) for 2 days and then analyzed using a dual luciferase assay to identify those with optimal basal and induced β-catenin activity.

Dual Luciferase Assay—A dual luciferase assay was carried out according to the manufacturer’s suggestions (Promega). RK3E and 293T cells were transfected with test plasmids of interest along with the β-catenin-LEF firefly luciferase reporter plasmid and the thymidine kinase-Renilla luciferase control plasmid. Two days post-transfection, the cells were harvested and assayed for firefly and Renilla luciferase activities using a Dual-Luciferase Reporter assay (Promega) according to the manufacturer’s suggestions. Briefly, the cells were lysed with 1× passive lysis buffer, and 10 μl of each cell lysate sample was transferred to a 96-well plate. 100 μl of luciferase assay reagent II was first injected to each well to measure the firefly luciferase activity followed by injection of 100 μl of Stop and Glow reagent for the measurement of the Renilla luciferase activities using a MicroLumat LB960 (Wallac).
RESULTS

Construction of Regulated β-Catenin Proteins—To study the role of activated β-catenin in signal transduction and cellular transformation we developed a system in which the activity of β-catenin could be rapidly and conditionally regulated. For this purpose an ER fusion strategy was utilized where the HBD of ER is fused to a protein of interest, thereby creating an estrogen-regulated protein activity (61). These ER fusion proteins are generally inactive and can be induced by estrogen or synthetic steroids such as 4-HT. Although not applicable to all proteins, such a strategy has been successful in generating a variety of functionally hormone-dependent proteins including cytoplasmic enzymes (Sre, Raf, and MAP kinase kinase) (62, 63) and transcription factors (Myc and LEF) (64–66).

ER-β-catenin fusion constructs were made by designing expression vectors in which sequences encoding an activated form of β-catenin, with a serine to alanine substitution at positions 37 and 45 (β-catenin S37A/A45A), were linked in-frame to sequences encoding a modified ER HBD (ER-G525R), which is unresponsive to estrogen yet can still be specifically activated by a synthetic estrogen, 4-HT (59). Previous studies have shown that the altered specificity of ER-G525R can prevent constitutive activation of ER fusion proteins by estrogen and/or estrogen agonists present in culture media (59). Because the functional effects of creating a hybrid protein between ER and β-catenin were unknown, two different chimeras were generated to place the ER sequences at either the N terminus (ER-β-catenin) or at the C terminus (β-catenin-ER) of β-catenin. Both versions were engineered to contain a N-terminal influenza HA epitope tag and were subcloned into a mammalian expression vector with a cytomegalovirus promoter-enhancer (Fig. 1A). To verify protein expression from these constructs, 293T cells were transfected using standard methods, and 2 days later cell extracts were examined by Western blot analysis using anti-HA antibody. Both constructs expressed β-catenin fusion proteins of expected size (Fig. 1B).

Induction of a β-Catenin-LEF-responsive Reporter Gene by Activation of ER-β-Catenin and β-Catenin-ER with 4-HT—To determine whether the ER-β-catenin and β-catenin-ER fusion proteins still retained transcriptional activity that could now be regulated by 4-HT, these proteins were analyzed using a dual luciferase reporter assay. A β-catenin-LEF-responsive reporter gene, similar to those described by others (67), was included at the N terminus of each protein. Several domains in β-catenin are indicated including the two transactivation domains (TA) located at the N and C termini. The armadillo repeats are represented as shaded boxes. The regions involved in binding to α-catenin, APC, axin, and cadherin are indicated. B, β-catenin protein expression. Plasmids encoding β-catenin S37A/A45A or the fusion protein ER-β-catenin or β-catenin-ER were transfected into 293T cells. Two days post-transfection, total cell extracts were prepared followed by Western blot analysis using a monoclonal antibody directed against the N-terminal HA tag.

by both β-catenin-ER and ER-β-catenin by ~5.5 and 9-fold, respectively. As expected, the ability of the activated β-catenin-positive control protein to stimulate β-catenin-LEF-dependent transcription was constitutive and independent of 4-HT. The level of reporter activity induced by 4-HT in cells expressing ER-β-catenin is comparable with that in cells expressing constitutively active β-catenin. Similar results were obtained using 293T cells (data not shown). In conclusion, the two fusion proteins ER-β-catenin and β-catenin-ER exhibit 4-HT-dependent ability to regulate β-catenin-LEF-dependent transcription.

Generation of Stable RK3E Cell Lines Expressing ER-β-Catenin—To perform additional biochemical and biological experiments utilizing the inducible β-catenin system it was desirable to create stable transfected cell lines. For this purpose we chose RK3E cells, a cell type that is known to respond to β-catenin signal transduction (68). Because ER fused to the N terminus of β-catenin consistently gave a slightly greater activation of the β-catenin-LEF reporter in transient transfection assays, this construct was used to generate stable transfected clones of RK3E cells. Approximately 60 stable cell clones were isolated after G418 selection and tested for regulated β-catenin-LEF-reporter activity after transient transfection with the reporter constructs. Four stable RK3E clones reproducibly exhibited low basal activity and 4-HT-dependent activation of the reporter gene (Fig. 3A). In the presence of 4-HT, clones 31 and 34 activated the reporter by ~5-fold, clone 96 by 9-fold, and clone 27 by 21-fold. Western blot analysis showed that the ER-β-catenin protein expressed by clone 31 was barely detectable (a longer exposure revealed a very low level of ER-β-catenin; data not shown), clone 34 and 96 had readily detectable expression, and clone 27 had the highest expression of ER-β-catenin (Fig. 3B). Although the basal levels of reporter activation are low...
Because ER-/H9252 able to activate a

A dual luciferase reporter assay was used for quantita-

tive analysis of β-catenin activity. A, regulated activation of a β-cate-

nin-LEF reporter by ER-β-catenin and β-catenin-ER. RK3E cells were

transfected with expression plasmids encoding ER-β-catenin, β-caten-
in-ER, β-catenin S37A/A45A, or Gal4 (control) along with the two

reporter plasmids outlined in A. The cells were then cultured in the

absence or presence of 1 μM 4-HT. Two days post-transfection, the cells

were harvested and analyzed for luciferase activity. The firefly lucifer-

ase activity was normalized for transfection efficiency using the Renilla

luciferase activity. The data represent an average of triplicate experi-

ments, and values are expressed as fold activation relative to the

negative control (Gal4).

with all clones, the degree of reporter activation upon 4-HT

treatment is higher in clones with higher levels of ER-β-catenin

protein expression. The Western blot analysis also revealed

that the level of ER-β-catenin protein was increased slightly

after 4-HT treatment, but this increase was not sufficient to

take into account for all of the increase in transcriptional activity (Fig.

3A). 4-HT had no discernible effects on the endogenous β-cate-

nin protein levels as determined by Western blot analysis (Fig.

3B). Additional experiments showed a dose response to 4-HT

for reporter gene activation in all clones with a concentration of

1 μg giving an optimal response (data not shown).

Induction of Endogenous Gene Expression by ER-β-Cate-
nin—Because ER-β-catenin, upon induction with 4-HT, was

able to activate a β-catenin-LEF-responsive reporter gene it

was also of interest to test whether this protein could activate

expression of a known cellular target gene. For this purpose we

chose to analyze cyclin D1, a well defined target gene for

β-catenin signaling in several cell types (53, 54). A TaqMan

quantitative PCR assay was established and used to measure

cyclin D1 levels in RK3E cells (clone 27) expressing ER-β-cate-
nin, either with or without 4-HT treatment for 1 or 2 days.

After 1 day of 4-HT treatment a 2-fold increase in cyclin D1

RNA was detected, and this increased to be ~5-fold after 2 days

(Fig. 3C). 4-HT treatment of RK3E control cells did not lead to

an increase in cyclin D1 levels (Fig. 3C). RK3E cells expressing

unfused β-catenin S37A/A45A were also tested in the experi-

ment in which cyclin D1 expression was examined. However, in this

case an increase in cyclin D1 RNA was not detected presumably

because of insufficient β-catenin levels to induce a measurable change in cyclin D1 (data not shown). We also noted that with RK3E cells where the activated ER-β-catenin signal was weaker, induction of cyclin D1 was not measurable (data not shown). Thus, high levels of activated β-catenin, including ER-β-catenin as shown here, are capable of inducing cyclin D1 RNA.

Association of ER-β-Catenin with Cadherin and α-Cate-
nin—In addition to its transcriptional function, β-catenin also

normally associates with cadherins and with α-catenin and thereby participates in the regulation of cell-to-cell adhesion.
We next examined whether the ER-\(\beta\)-catenin protein was also able to form a complex with these proteins and whether this could be modulated by 4-HT. Control cells or ER-\(\beta\)-catenin-expressing cells, clones 27 and 96, were incubated for 2 days in the presence or absence of 4-HT. Equivalent amounts of cell extracts were immunoprecipitated with either an antibody directed against \(\alpha\)-catenin or against E-cadherin, and the immunoprecipitates were subjected to Western blot analysis with an antibody against \(\beta\)-catenin. Both ER-\(\beta\)-catenin and endogenous \(\beta\)-catenin were complexed with \(\alpha\)-catenin, and this was relatively unchanged in the presence of 4-HT (Fig. 4A). Although the total level of endogenous \(\beta\)-catenin was comparable with the total level of ER-\(\beta\)-catenin, the endogenous \(\beta\)-catenin predominated in complex with cadherin, and this was not affected by 4-HT treatment (Fig. 4B). These data indicate that although the ER-\(\beta\)-catenin protein is capable of associating with cadherin, the endogenous \(\beta\)-catenin protein is the predominant form found in complex with cadherin. This suggests that the ER domain of ER-\(\beta\)-catenin may interfere with its ability to bind to cadherin. In any case, the association of endogenous \(\beta\)-catenin with cadherin is not affected by the presence of ER-\(\beta\)-catenin, with or without the addition of 4-HT.

To evaluate further the subcellular distribution of ER-\(\beta\)-catenin relative to endogenous \(\beta\)-catenin, the ER-\(\beta\)-catenin cells (clones 27 or 96) or control cells were separated into Nonidet P-40-soluble and -insoluble fractions as described under “Experimental Procedures.” The Nonidet P-40-insoluble fraction is likely to consist of a variety of subcellular components including cytoskeleton and nucleus. The two fractions from each cell type were subjected to Western immunoblot analysis with an antiserum against \(\beta\)-catenin. Endogenous \(\beta\)-catenin was identified in both the soluble and insoluble fractions in approximately equal amounts, and this was unchanged by the addition of 4-HT (Fig. 4C). In the absence of 4-HT, ER-\(\beta\)-catenin was localized to both the soluble and insoluble fractions (Fig. 4C). Upon addition of 4-HT a majority of ER-\(\beta\)-catenin was shifted to the insoluble fraction (Fig. 4C).

**Nuclear Translocation of ER-\(\beta\)-Catenin in the Presence of 4-HT**—To examine the subcellular localization of the ER-\(\beta\)-catenin fusion protein in more detail, immunofluorescence studies were performed with a stable RK3E clone expressing the highest levels of ER-\(\beta\)-catenin (clone 27) and a control RK3E clone (clone 152) with no expression of ER-\(\beta\)-catenin. The cells were plated on coverslips and then cultured in medium with or without 1 \(\mu\)M 4-HT for either 1 or 2 days. The cells were fixed and stained either with one monoclonal antibody directed against \(\beta\)-catenin (A) or HA tag (B) or E-cadherin (C). The antibody staining was visualized by fluorescence microscopy using a Nikon inverted microscope (Eclipse TE300) with a 40× objective.

**FIG. 4. Complex formation and subcellular distribution of ER-\(\beta\)-catenin.** RK3E cells expressing ER-\(\beta\)-catenin (clones 27 and 96) and a control clone with no expression of ER-\(\beta\)-catenin were grown with or without 1 \(\mu\)M 4-HT for 2 days. A and B, cell extracts were prepared, and the Nonidet P-40-soluble fraction of each was immunoprecipitated with an antibody against either \(\alpha\)-catenin or E-cadherin, and clone 27 extracts were also immunoprecipitated with a control antibody (NIS). Washed immunoprecipitates were analyzed by Western immunoblot with an antibody against \(\beta\)-catenin. C, Nonidet P-40-soluble and - insoluble fractions were prepared from the same cells as used in A and B. Equivalent aliquots of total protein from each fraction were analyzed by Western immunoblot with an antibody against \(\beta\)-catenin.

**FIG. 5. Immunofluorescence localization of \(\beta\)-catenin and E-cadherin in cells expressing ER-\(\beta\)-catenin.** RK3E clone 27, which expresses ER-\(\beta\)-catenin, and clone 152, a control with no expression of ER-\(\beta\)-catenin, were grown on coverslips and then cultured in medium with or without 1 \(\mu\)M 4-HT for either 1 or 2 days. The cells were fixed and stained with either a monoclonal antibody directed against \(\beta\)-catenin (A) or HA tag (B) or E-cadherin (C). The antibody staining was visualized by fluorescence microscopy using a Nikon inverted microscope (Eclipse TE300) with a 40× objective.
localized. Activation of the β-catenin-LEF reporter was first detectable between 1 and 2 h after 4-HT treatment suggesting that nuclear translocation and functional activation of the ER-β-catenin protein is rapid (data not shown).

**Down-regulation of E-cadherin by β-Catenin—Immunofluorescence experiments were also carried out with a monoclonal antibody specific for E-cadherin. Evaluation of the control cells showed the typical membrane staining pattern characteristic of cadherins (69, 70), and this was unaffected by the addition of 4-HT (Fig. 5C). The cadherin staining pattern of the untreated clone 27 ER-β-catenin-expressing cells was somewhat weaker than that of the control cells (Fig. 5C). However, upon addition of 4-HT to the clone 27 cell line the specific cadherin staining was greatly diminished by 2 days (Fig. 5C). To follow up on this observation, the levels of cadherin protein were also examined by Western immunoblot analysis using antibodies directed against E-cadherin. The level of E-cadherin protein was lower in untreated clone 27 cells when compared with the untreated control or clone 96 cells. Treatment of clone 27 or 96 ER-β-catenin cells for 2 days significantly decreased the level of E-cadherin protein while having no discernible effects on a control clone that did not express ER-β-catenin (Fig. 6). These data suggest that β-catenin signaling can down-regulate cadherin expression. The diminished cadherin levels in clone 27 the absence of 4-HT may be the result of the small amount of basal activity of the ER-β-catenin in these untreated cells. Because it has been reported that activation of β-catenin signaling can regulate the expression of keratin and vimentin (71, 72), we also examined by Western immunoblot whether ER-β-catenin can regulate the levels of these two structural proteins. Both keratin and vimentin proteins were readily detectable in the RKE cells, but the level of neither protein was changed upon activation of ER-β-catenin by 4-HT (Fig. 6).

**Protection against Anoikis by β-Catenin—**We noted that overnight 4-HT treatment of clones exhibiting the highest β-catenin-LEF reporter activity upon induction (clones 27 and 96), but not control clones, led to a loss of adhesion of many of these cells to the tissue culture dish (Fig. 7A). Approximately 50% of the detached cells from clone 96 reattached upon plating in the absence of 4-HT (data not shown). Clone 27 or 96 cells plated on matrigel still showed the same tendency to detach in the presence of 4-HT (data not shown). To determine whether the detached cells from 4-HT-treated ER-β-catenin clones were viable or apoptotic, floating cells were harvested from subconfluent monolayer cultures of untreated and 5-day 4-HT-treated clone 27 and 96 cells. As an additional control, the few detached cells were harvested from a control clone that did not express ER-β-catenin, with or without 4-HT treatment. Detached cells were then stained with a monoclonal antibody against annexin V, an apoptosis marker (73), followed by FACS analysis. A majority of detached cells harvested from the control clone with or without 4-HT treatment (76 and 77%, respectively) or from untreated clones 27 and 96 (51 and 60%, respectively) were apoptotic (Fig. 7B). In contrast, a minority of the detached cells from 4-HT-treated clones 27 and 96 (28 and 38%, respectively) were apoptotic. Consequently, treatment of clones 27 and 96 to induce ER-β-catenin activity increased the percentage of viable cells in suspension by 45 and 55%, respectively (Fig. 7C).

The findings shown in Fig. 7 imply that β-catenin can inhibit anoikis resulting from loss of cell-substrate contact. To test this hypothesis, four ER-β-catenin-expressing clones and one negative control clone were cultured in tissue culture plates coated with poly-HEME, which prevents cell attachment. Suspended cells were then analyzed for viability after 18 h with or without 4-HT treatment. In the presence of 4-HT, all four clones expressing ER-β-catenin exhibited a higher percentage of viable
cells (20–50% more) when compared with the untreated cells (Fig. 8, A and B), suggesting that activation of ER-β-catenin can rescue these cells from anoikis. The negative control RK3E clone, expressing no exogenous β-catenin, showed essentially no increase in viable cells upon 4-HT treatment (Fig. 8, A and B). As a positive control, RK3E cells infected with a retrovirus expressing constitutively activated β-catenin, S37A/A45A were also analyzed. Similar to activated ER-β-catenin fusion protein, β-catenin S37A/A45A also increased the percentage of viable cells by −54% (Fig. 8B).

**Blocking MAP Kinase Signaling Abrogates Protection from Anoikis by β-Catenin**—MAP kinase signaling has been implicated in promoting both cell proliferation and cell survival (74, 75). Therefore, we tested whether protection against anoikis by β-catenin is dependent on the MAP kinase pathway. PD98059 is a potent inhibitor of MAP kinase kinase (76) and has been widely used to inhibit the activation of MAP kinase. An anoikis assay was carried out as described above in the presence or absence of PD98059. As expected, the addition of 4-HT to RK3E cells expressing ER-β-catenin increased the percentage of viable cells in suspension by −50% (Fig. 9, A and B). However, PD98059 completely abolished this protective effect (Fig. 9, A and B), suggesting that MAP kinase signaling is involved in the rescue of cells from anoikis by β-catenin.

**Activation of MAP Kinase by β-Catenin**—Because the MAP kinase pathway appeared to be involved in protection against anoikis by β-catenin, we next assessed whether MAP kinase itself is activated by β-catenin induction. RK3E cells expressing ER-β-catenin or a control clone were cultured in medium with or without 4-HT. Cells were analyzed either as monolayer cultures or in suspension under anoikis assay conditions. After overnight growth and treatment, cells were harvested and extracts were analyzed by Western blot using antibodies directed against phosphorylated p42/p44 MAP kinase (top). A duplicate blot was probed with an antibody directed against MAP kinase (bottom). A similar experiment with the same cell types was performed with cells plated on poly-HEMA-coated plates instead of on plastic (right panels). After 18 h in suspension, cell extracts were prepared and analyzed by Western blot using antibodies directed against phosphorylated p42/p44 MAP kinase (top). A duplicate blot was probed with an antibody directed against MAP kinase (bottom).

**Fig. 8. β-Catenin protects RK3E cells from anoikis.** Four RK3E cell lines expressing ER-β-catenin (clones 27, 31, 34, and 96), a negative control RK3E cell line (clone 152), and positive control RK3E cells infected with a retrovirus coexpressing β-catenin S37A/S45A and green fluorescence protein were trypsinized into a single cell suspension, and 2.5 ml was cultured poly-HEMA-coated plates at a density of ~10^6 cells/ml (total of 2.5 × 10^6 cells) in the absence or presence of 1 μM 4-HT and then incubated at 37°C for an additional 18 h. Cells were stained with annexin V-phycocerythrin antibodies and analyzed by FACS. A, representative data obtained by FACS analysis of two ER-β-catenin-expressing cell lines (clones 34 and 96) and a control cell line (clone 152). B, plot of the percentage increase in viable cells induced by 4-HT in the four ER-β-catenin-expressing clones, a negative control clone, and positive control cells expressing β-catenin S37A/S45A from a separate experiment.

**Fig. 9. Role of MAP kinase in β-catenin-induced protection from anoikis.** An anoikis assay was carried out in the presence or absence of 50 μM PD98059 (Calbiochem) using cells expressing ER-β-catenin (clone 34) or control cells (clone 152). Cells were then stained with anti-annexin V antibodies and analyzed by FACS (A). B, plot of the percentage change in viable cells based on FACS data from A. C, left panels, RK3E cells expressing ER-β-catenin (clone 27) or control cells (clone 152) were cultured on tissue culture plastic in growth medium with or without 4-HT for 2 days. The cells were then harvested, and extracts were analyzed by Western blot using antibodies directed against phosphorylated p42/p44 MAP kinase (top). A duplicate blot was probed with an antibody directed against MAP kinase (bottom). A similar experiment with the same cell types was performed with cells plated on poly-HEMA-coated plates instead of on plastic (right panels). After 18 h in suspension, cell extracts were prepared and analyzed by Western blot using antibodies directed against phosphorylated p42/p44 MAP kinase (top). A duplicate blot was probed with an antibody directed against MAP kinase (bottom).
DISCUSSION

To create a regulated system for the study of β-catenin signaling, we constructed a conditionally active β-catenin protein. The activity of this hybrid protein (ER-β-catenin), with the ER HBD fused to a stabilized form of β-catenin, could be rapidly induced upon addition of the synthetic estrogen 4-HT in a dose-dependent fashion. Expression of ER-β-catenin by both transient and stable transfection led to transcriptional activation of a LEF/β-catenin-responsive reporter gene in the presence of 4-HT. 4-HT-dependent activation of ER-β-catenin was also able to induce expression of cyclin D1, a previously defined cellular target gene for β-catenin signaling (53, 54).

Treatment with 4-HT led to a small increase in the steady-state level of the ER-β-catenin protein, but this is unlikely to account for the dramatic induction of transcriptional activity seen upon the addition of 4-HT. This is similar to the results of another study where the level of a Raf-ER fusion protein increased almost 10-fold after 16 h of estradiol or 4-HT treatment (63). It appears that ligand-induced changes in subcellular localization and protein conformation may influence the stability of fusion proteins.

Immunofluorescence experiments showed that most of the ER-β-catenin was distributed diffusely throughout the cytoplasm in the absence of 4-HT but was translocated efficiently into the nucleus upon addition of 4-HT. In the presence of 4-HT most of the ER-β-catenin protein was nuclear in contrast to the distribution of activated β-catenin in other cell types where significant cytoplasmic and membrane populations are present (58). This could be the result of a strong cooperation between the portion of β-catenin that enables nuclear localization (77) and the fused ER domain or caused by compromised nuclear export of the hybrid protein once it is in the nucleus. Several other recombinant proteins with ER fused to nuclear proteins such as transcription factor GATA-1, DNA repair methyltransferase, and T cell leukemia/lymphoma virus type 1 Rex protein (78–80) also exhibited hormone-dependent nuclear translocation. It is likely that the addition of hormone is permissive for subcellular localization defined by the protein component fused to the ER domain.

The predominantly cytoplasmic (−4-HT) or nuclear (+4-HT) localization of ER-β-catenin, described by immunofluorescence, is consistent with biochemical fractionation and immunoprecipitation studies using the same cells. Based on coimmunoprecipitation studies, the ER-β-catenin protein was able to complex with α-catenin as expected. However, very little ER-β-catenin was found associated with cadherin compared with endogenous β-catenin, despite comparable expression levels of these two catenin proteins. The compromised association of ER-β-catenin with cadherin could be caused by steric hindrance by the ER portion of the protein or interference by other associated proteins. The cadherin-ER-β-catenin or cadherin-endogenous β-catenin complexes were not affected by 4-HT treatment. The predominantly cytoplasmic ER-β-catenin, observed in the absence of 4-HT, appeared to shift into a Nonidet P-40-insoluble cell fraction upon addition of 4-HT, presumably reflecting the shift to a predominantly nuclear localization seen by immunofluorescence.

The mechanism by which β-catenin is normally translocated into the nucleus is not well understood. β-Catenin lacks a classic nuclear localization sequence, but the armadillo repeats at the C terminus are both necessary and sufficient to confer nuclear translocation (81, 82). These repeats share structural resemblance with the tandem repeats of importin-β, which facilitate nuclear import by direct binding to the nuclear pore machinery (77, 83, 84). With respect to the ER-β-catenin protein, the diffuse cytoplasmic localization in the absence of hormone may be the result of its poor association with cadherin and may also be a consequence of its association with Hsp90 or other proteins that are known to complex with the ER and render it inactive (85, 86). Binding of 4-HT to ER-β-catenin may expose functional domains in β-catenin involved in nuclear import and thus facilitate its nuclear localization. The results presented here show that transcriptional activation by ER-β-catenin is only evident under conditions in which the protein is localized to the nucleus. These data suggest models proposed by others in which the transcriptional activity of β-catenin is dependent on its transport to the nucleus, which can be regulated by association with cadherin or sequestration by other means (87, 88).

Immunofluorescence studies and Western blot analysis showed that the activation of ER-β-catenin by 4-HT correlates with a decrease in cadherin expression. Because the endogenous β-catenin is the predominant form found in association with cadherin and because ER-β-catenin binds well to α-catenin, it is unlikely that the small amount of ER-β-catenin associated with cadherin is leading to changes in cadherin protein levels. The decreased cadherin expression in response to activation of ER-β-catenin extends other observations that implicate the β-catenin signaling pathway in the regulation of E-cadherin levels (71). Overexpression of integrin-linked kinase leads to a down-regulation of the level of E-cadherin protein (44, 89). Integrin-linked kinase is thought to facilitate β-catenin signaling, and the cells overexpressing integrin-linked kinase show strong nuclear localization of β-catenin (44). The promoter of the E-cadherin gene (CDH1) contains consensus binding sites for the β-catenin-LEF transcription factor complex, and it has been proposed that this complex can down-regulate the expression of CDH1 (90, 91). However, the levels of cadherin mRNA are very low in RK3E cells, and we were unable to detect changes because of activation of the ER-β-catenin protein (data not shown). Members of the cadherin family of cell adhesion molecules are expressed broadly and play essential roles in regulating normal cell adhesion, migration, and differentiation (90, 92). Multiple lines of evidence suggest that E-cadherin can function as a tumor suppressor, and down-regulation or loss of expression of E-cadherin has been observed in a wide variety of tumors (91, 93–99). The diminished expression of E-cadherin in tumors correlates with an epithelial-mesenchymal transition, increased tumor cell invasion, migration, and metastasis (100, 101), whereas the invasive phenotype of epithelial tumor cells can be suppressed after restoration of E-cadherin expression (102). Based on these data it has been proposed that loss of E-cadherin expression is a rate-limiting step during tumor progression (103). In normal cells β-catenin binding to the cytoplasmic tail of cadherin is known to regulate cadherin-mediated cell-to-cell adhesion (104). β-Catenin has also been implicated in epithelial-mesenchymal transition, cell migration, and other phenotypes of malignant transformation (34, 35, 42). Consequently, the elevated level of β-catenin signaling in tumors and the ability of β-catenin to down-regulate cadherin expression may contribute significantly to tumor progression. Down-regulation of cadherin by β-catenin signaling may also serve as a positive feedback loop because reduced cadherin levels could lead to an increase in the free pool of β-catenin and thereby increase β-catenin-LEF-dependent transcriptional activation of relevant target genes that promote transformation independent of cadherin expression or function.

Survival of normal epithelial cells is dependent on their interactions with extracellular matrix, and when deprived of such interactions, they undergo a form of programmed cell death termed anoikis (105–107). This process prevents reat-
tachment and growth of epithelial cells that have lost adherence to the extracellular matrix and plays a critical role in maintaining the balanced process of proliferation and turnover in epithelium. Resistance to apoptosis and anoikis is a common feature of many cancers and contributes to tumor progression and chemoresistance (106, 108, 109). Stable RK3E cell lines expressing ER-β-catenin exhibit a marked reduction in anoikis upon activation by 4-HT. This finding supports other reports that implicate β-catenin signaling in the regulation of apoptosis. Restoration of APC expression in tumor cells with nonfunctional APC led to apoptosis (110) presumably by facilitating the down-modulation of β-catenin. Stable overexpression of β-catenin has been shown to inhibit anoikis in Madin-Darby canine kidney-derived epithelial cells (111), whereas β-catenin was cleaved by caspase-3 during apoptosis (112). In another study, Wnt-1, which activates β-catenin signaling, inhibited chemotherapeutic drug-induced apoptosis in Rat-1 cells by a process that was dependent on the activation of β-catenin-LEF-mediated transcription (113).

The mechanism by which activated β-catenin can inhibit apoptosis or anoikis is not well defined. In one study plakoglobin, another catenin family member, was shown to regulate Bcl-2 protein levels (data not shown). The experiments with ER-β/catenin did not detect any effects of 4-HT induction on Bcl-2 and inhibit apoptosis or anoikis (115). Factors and by integrin engagement to promote proliferation hepatocyte growth factor, which activate MAP kinase, can co-regulated kinase 1/2) is activated upon induction of ER-β-catenin signaling, inhibited chemoresistance (106, 108, 109). Stable RK3E cell lines containing the balanced process of proliferation and turnover in epithelium. Resistance to apoptosis and anoikis is a common feature of many cancers and contributes to tumor progression and chemoresistance (106, 108, 109). Stable RK3E cell lines expressing ER-β-catenin exhibit a marked reduction in anoikis upon activation by 4-HT. This finding supports other reports that implicate β-catenin signaling in the regulation of apoptosis. Restoration of APC expression in tumor cells with nonfunctional APC led to apoptosis (110) presumably by facilitating the down-modulation of β-catenin. Stable overexpression of β-catenin has been shown to inhibit anoikis in Madin-Darby canine kidney-derived epithelial cells (111), whereas β-catenin was cleaved by caspase-3 during apoptosis (112). In another study, Wnt-1, which activates β-catenin signaling, inhibited chemotherapeutic drug-induced apoptosis in Rat-1 cells by a process that was dependent on the activation of β-catenin-LEF-mediated transcription (113).

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