[CANCER RESEARCH 61, 1671–1677, February 15, 2001]

Geldanamycin Abrogates ErbB2 Association with Proteasome-resistant β-Catenin in Melanoma Cells, Increases β-Catenin-E-Cadherin Association, and Decreases β-Catenin-sensitive Transcription

Paolo Bonvini, Won G. An, Angelo Rosolen, Phongmai Nguyen, Jane Trepel, Antonio Garcia de Herreros, Mireia Dunach, and Leonard M. Neckers[1]

Department of Cell and Cancer Biology, Medicine Branch, National Cancer Institute, Rockville, Maryland 20850 [P. B., W. G. A., P. N., J. T., L. M. N.]; Oncology and Hematology Branch, Clinic of Pediatrics, University of Padova, Padova 35128, Italy [P. B., A. R.]; Institut Municipal d'Investigacio Medica, Universitat Pompeu Fabra, Barcelona 08003, Spain [G. H.]; and Departament de Bioquimica i Biologia Molecular, Universitat Autonoma de Barcelona, Bellaterra 08193, Spain [M. D.]

ABSTRACT

β-catenin undergoes both serine and tyrosine phosphorylation. Serine phosphorylation in the amino terminus targets β-catenin for proteasome degradation, whereas tyrosine phosphorylation in the COOH terminus influences interaction with E-cadherin. We examined the tyrosine phosphorylation status of β-catenin in melanoma cells expressing proteasome-resistant β-catenin, as well as the effects that perturbation of β-catenin tyrosine phosphorylation had on its association with E-cadherin and on its transcriptional activity. β-catenin is tyrosine phosphorylated in three melanoma cell lines and associates with both the ErbB2 receptor tyrosine kinase and the LAR receptor tyrosine phosphatase. Geldanamycin, a drug which destabilizes ErbB2, caused rapid cellular depletion of the kinase and loss of its association with β-catenin without perturbing either LAR or β-catenin levels or LAR/β-catenin association. Geldanamycin also stimulated tyrosine dephosphorylation of β-catenin and increased β-catenin/E-cadherin association, resulting in substantially decreased cell motility. Geldanamycin also decreased the nuclear β-catenin level and inhibited β-catenin-driven transcription, as assessed using two different β-catenin-sensitive reporters and the endogenous *cyclin D1* gene. These findings were confirmed by transient transfection of two β-catenin point mutants, Tyr-654Phe and Tyr-654Glu, which, respectively, mimic the dephosphorylated and phosphorylated states of Tyr-654, a tyrosine residue contained within the β-catenin-ErbB2-binding domain. These data demonstrate that the functional activity of proteasome-resistant β-catenin is regulated further by geldanamycin-sensitive tyrosine phosphorylation in melanoma cells.

INTRODUCTION

β-catenin is a multifunctional protein that plays an important role in both cell-cell interactions (1, 2) and transcriptional regulation (3–6). In normal epithelial cells, β-catenin associates with the cytoplasmic domain of E-cadherin, linking this transmembrane adhesion molecule to the actin cytoskeleton via hydrophobic association with α-catenin (7–11). β-catenin-E-cadherin association is critically important for the maintenance of tight cell-cell contacts (12, 13). When released from E-cadherin, uncomplexed β-catenin is rapidly degraded by cytosolic proteasomes (14, 15). Failure to properly degrade β -catenin, primarily attributable to an impairment in its ubiquitination, results in β-catenin accumulation and migration to the nucleus where, via interaction with the Tcf/Lef family of transcription factors, it can up-regulate transcription of a number of growth-promoting genes, including c-*myc* and *cyclin D* (16–19). In a large number of cancers, including colon carcinoma and melanoma, proteasomal degradation of cytoplasmic β-catenin is subverted, either by mutation of a GSK-3β[2] phosphorylation site in the amino terminus of β-catenin or by deletion of the APC β-catenin-binding protein (20–22). The resultant nuclear accumulation of β-catenin is thought to play a pivotal role in tumor progression (23–27).

Although many studies have focused on mechanisms that abrogate the proteasome sensitivity of uncomplexed cytoplasmic β-catenin in tumor cells, less attention has been paid to the status and regulation of β-catenin-E-cadherin association in cells that either express GSK-3β-resistant β-catenin or lack the APC protein. In normal epithelial cells, association of the cadherin-catenin complex with the cytoskeleton is essential for maintenance of functional epithelial tissues (28), but dynamic regulation of this complex is necessary for the epithelial migration characteristic of embryogenesis and wound healing (29). β-catenin-E-cadherin association/dissociation seems to be regulated, at least in part, by tyrosine phosphorylation/dephosphorylation of β-catenin (28), inasmuch as tyrosine phosphorylated β-catenin associates poorly, if at all, with E-cadherin (30). During normal epithelial migration, β-catenin has been shown to accumulate in the cytoplasm in a free, tyrosine-phosphorylated form concomitant with increased nuclear localization of β-catenin (28, 31, 32). In contrast, in confluent cells, β-catenin is found primarily in a tyrosine-dephosphorylated form and in complex with E-cadherin. Because the loss of β-catenin-E-cadherin association correlates directly with tumor invasion and metastasis, a second level of β-catenin deregulation in tumor cells, particularly in situations where β-catenin degradation is already compromised, might result from an imbalance in the tyrosine phosphorylation state of β-catenin (33).

In a recent report, ErbB2 (Her2/Neu) tyrosine kinase overexpression was observed in 40% of cutaneous malignant melanomas and in 80% of metastasized malignant melanomas (34), whereas a second study found ErbB2 protein to be overexpressed in four of five melanoma cell lines (35). Interestingly, ErbB2 as well as several transmembrane tyrosine phosphatases, including PTPμ, PTPκ, PTPλ, and LAR, have been found to be associated with β-catenin-E-cadherin complexes in epithelial cells, suggesting opposing roles for these proteins in modulating β-catenin-E-cadherin association (28, 36–42). ErbB2 interacts with the carboxyl terminus of β-catenin, at a site containing Tyr-654 (43). Recently, Roura *et al.* (30) reported that the Tyr-654 residue of β-catenin is preferentially phosphorylated under conditions that disrupt β-catenin-E-cadherin association. Furthermore, these investigators observed that a Tyr-654Phe (Y654F) point mutant, which mimics an unphosphorylated tyrosine residue, bound E-cadherin *in vitro* with an affinity similar to that of wild-type β-catenin, whereas a Tyr-654Glu (Y654E) point mutant, whose negative charge mimics a phosphorylated tyrosine residue, bound E-cadherin with less than one-tenth the affinity of wild-type β-catenin. Thus, it is reasonable to conclude that the phosphorylation status of

Received 6/27/00; accepted 12/12/00.

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[1] To whom requests for reprints should be addressed, at: 9610 Medical Center Drive, Suite 300, Rockville, MD 20850. Phone: (301) 402-3128, extension 318; Fax: (301) 402-4422; E-mail: len@helix.nih.gov.

[2] The abbreviations used are: GSK-3β, glycogen synthase kinase-3β; APC, adenomatous polyposis coli; GA, geldanamycin; HA, hemaglutinin.

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the Tyr-654 residue of β-catenin, contained within an ErbB2-binding domain, regulates β-catenin association with E-cadherin.

Preliminary characterization of the three melanoma cell lines used in this study demonstrated constitutive expression of both ErbB2 and LAR. Here, we have examined the tyrosine phosphorylation state of β-catenin and its association with E-cadherin, ErbB2, and LAR in these cell lines. These cell lines were chosen for study because one, 1011, expresses β-catenin, which is recognized and degraded normally by cytosolic proteasomes. Another, 928, expresses wild-type β-catenin but lacks the APC protein. A third cell line, 1241, expresses APC but contains β-catenin whose GSK-3β amino terminal phosphorylation site is mutated (Ser-37Phe). In the latter two cell lines, uncomplexed β-catenin is not degraded efficiently by the proteasome and accumulates to high levels in both cytoplasm and nucleus, whereas in 1011 cells the β-catenin steady-state level is low because of rapid proteasome-mediated degradation of uncomplexed β−catenin (22, 44). We used GA, a drug which efficiently stimulates the proteasome-mediated degradation of ErbB2 (45, 46), to assess its effects on the tyrosine phosphorylation state of β-catenin, on the association of both LAR and E-cadherin with β-catenin, and on β-catenin-sensitive transcriptional activity. Finally, we transiently transfected 1241 melanoma cells with the β-catenin point mutants Y654F and Y654E to confirm that our findings with GA are attributable to the altered tyrosine phosphorylation status of β-catenin. Our data demonstrate the importance of tyrosine phosphorylation in regulating β-catenin activity in melanoma cells regardless of whether these cells express proteasome-sensitive or -insensitive β−catenin.

MATERIALS AND METHODS

Antibodies and Other Reagents. Culture media were purchased from Biofluids, Inc. GA was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute. Anti-β-catenin monoclonal antibody was purchased from Transduction Labs. Anti-Hsp90 monoclonal antibody was obtained from StressGen. Anti-cyclin D and anti-E-cadherin antibodies were obtained from PharMingen. Anti-phosphotyrosine monoclonal antibody (PY20) and anti-ErbB2 monoclonal antibody (AB3) were obtained from Oncogene Science. Anti-LAR monoclonal antibody was a kind gift of Dr. Michel Streuli (Dana-Farber Cancer Institute, Boston, MA). Anti-HA antibody was purchased from Covance. Rabbit antimouse IgG1 was obtained from Cappel, and horseradish peroxidase-conjugated sheep antimouse antibody was purchased from Amersham Life Science. Protein A-Sepharose beads were purchased from Pharmacia. BCA Protein Assay reagent and Western blot chemiluminescence reagents were purchased from Pierce Chemical Co. Protran nitrocellulose membranes were obtained from Schleicher & Schuell. All of the other chemicals used in this study were purchased from Sigma Chemical Co.

Cell Culture. The melanoma cell lines 1011, 928, and 1241 (obtained from Mona El-Gamil and Paul Robbins, NIH, Bethesda, MD) were maintained in RPMI 1640 containing 5% bovine calf serum, 2 mM L-glutamine, and 10 mM HEPES (pH 7.5) under standard tissue-culture conditions.

Immunoprecipitation and Immunoblotting. Cells were washed twice in ice-cold PBS and incubated with ice-cold Triton X-100 lysis buffer [Tris-HCl (pH 8), 20 mM; Triton X-100, 1%; NaCl, 140 mM; Glycerol, 10%; EGTA, 1 mM; $MgCl_2$, 1.5 mM; DTT, 1 mM; sodium vanadate, 1 mM; and NaF, 50 mM] containing 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 20 μg/ml aprotinin. Cells were scraped after 15 min, and cell lysates were clarified by centrifugation (at 4°C) at 14,000 × *g* for 30 min. Immunoprecipitations from cell lysates, SDS-PAGE, electrotransfer, and immunoblotting were carried out as described previously. Proteins were visualized by chemiluminescence using a commercial kit (Pierce). X-OMAT AR films (Kodak) were scanned into a computer, and the images were quantified using image analysis software (NIH Image).

To analyze β-catenin in cytosolic and nuclear fractions, intact nuclei were prepared from exponentially growing 928, 1011, and 1241 cells by scraping cells into nuclei isolation buffer (100 mM NaCl, 10 mM Tris-HCl, 5 mM sodium butyrate, 10 mM iodoacetamide, 0.1% NP-40, and phosphatase inhibitors, as described above). Cell suspensions were gently homogenized in an ice-cold glass homogenizer. A cytosolic fraction (supernatant) was separated from intact nuclei by low-speed centrifugation at 4°C. To obtain final cytosolic fractions, these low-speed supernatants were subjected to high-speed centrifugation, and supernatants were isolated again. Intact nuclei, observed by staining with trypan blue, were then lysed in nuclei-isolation buffer supplemented with 1% Triton X-100. Aliquots of these samples were fractionated by 10% SDS-PAGE, electrotransferred to nitrocellulose membranes, and analyzed by Western blotting.

Immunofluorescence of ErbB2 and LAR. Melanoma cells (928) were grown on coverslips and exposed to GA (1 μM) for 14 h or left untreated. After being rinsed in PBS, cells were fixed in 3.7% formaldehyde in PBS and permeabilized with 0.2% Triton X-100. LAR and ErbB2 were visualized by immunofluorescence as described previously (47). The DNA-intercalating dye DAPI was used to identify cell nuclei.

Transient Transfections. To determine specific effects of nuclear accumulation of β-catenin on Tcf/Lef-dependent transcription, melanoma cells were transiently transfected with the reporter constructs pTOPFLASH and pFOPFLASH, which contain, respectively, three copies of the optimal (CCTTTGATC) or mutant (CCTTTGGCC) Tcf-binding motif upstream of a minimal c-Fos promoter-driving luciferase expression. A second Tcf/Lef-responsive reporter plasmid, 7Lef-fosLuc, was also used. For the transient transfection experiment shown in Fig. 4*D*, 1 μg of the reporter was transfected into 928 or 1241 cells (1×10^6 cells for each cell line) using lipofectamine, according to manufacturer's instructions (Life Technologies, Inc.). To avoid nonspecific, drug-dependent effects on luciferase expression, we performed a DUAL-Luciferase reporter assay (Promega). A control reporter, pRL-TK (0.5 μg), which contains a herpes simplex virus thymidine kinase promoter driving a *Renilla luciferase* gene, was cotransfected with the β-catenin-specific luciferase constructs, and Renilla luciferase activity was used to normalize the results. After 24 h, cells were lysed in Triton X-100 lysis buffer and luciferase activity was monitored in 30 μg of cell lysate using luciferase assay reagents as described by the manufacturer (Promega). β-catenin(Y654F) and β-catenin(Y654E) have been described previously (30). Cells (293 and 1241) were transfected as above and as described in the legend to Fig. 6.

Motility Assay. Medium (0.7 ml) was added to each well of a 24-well plate (Costar). Cell culture inserts (Fisher; 8 μm pore size, Falcon 3097) were placed into each well. Melanoma cells were adjusted to a concentration of 1.5×10^5 cells/ml, and 350 μl of cells were placed into each insert. Cells were incubated for 48 h with increasing concentrations of GA (0, 0.5, 1, and 2 μM). After 48 h, the number of cells adherent to the bottom of the well was counted. Motility is expressed as a percentage of the number of cells in drug-free wells adhering to the well after 48 h. Cells were identified for counting by Wright stain.

RESULTS

β-Catenin-E-Cadherin Association in Melanoma Is Inversely Related to β-Catenin Tyrosine Phosphorylation State. Melanoma cell lines (1011, 928, and 1241) were maintained in log phase growth. Cells were lysed in Triton X-100 lysis buffer and immunoprecipitated with a phosphotyrosine-specific antibody, an antibody specific for E-cadherin, or an antibody specific for β-catenin. After SDS-PAGE, all immunoblots were probed with a β-catenin-specific antibody (Fig. 1). Unlike normal epithelial cells, which must be treated with phosphatase inhibitors to detect tyrosine phosphorylated β-catenin (28), the three melanoma cell lines contained readily detectable tyrosine phosphorylated β-catenin in the absence of phosphatase inhibition (Fig. 1*A*, *Lane 1*). After exposure to GA, the level of tyrosine phosphorylated β-catenin declined markedly without any overall change in the β-catenin steady-state level (Fig. 1, *A* and *C*). The amount of β-catenin coimmunoprecipitated with E-cadherin dramatically increased as the proportion of tyrosine phosphorylated β-catenin declined (Fig. 1*B*), although total E-cadherin levels remained unchanged (Fig. 1*D*). Mutated (S37F) and wild-type β-catenin responded similarly; APC status also did not affect the results. Thus, abrogation of β-catenin tyrosine phosphorylation markedly enhances β-catenin-E-cadherin association even when β-catenin is resistant to proteasome degradation.

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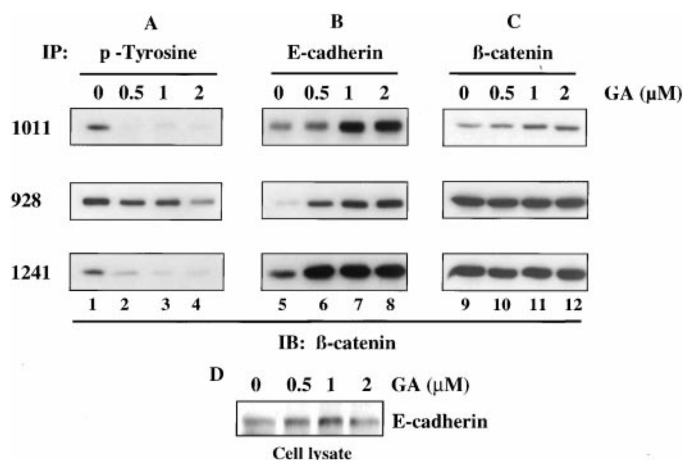

Fig. 1. Exponentially growing 1011, 928, and 1241 melanoma cells were cultured for 24 h in the presence of 0.5, 1, and 2 μM GA. Cytosolic fractions were separated from intact nuclei as described in "Materials and Methods." *A*, to detect tyrosine phosphorylated β-catenin, 1 mg of cytosol proteins was precipitated with 3 μg of anti-P-tyrosine antibody (*Lanes 1–4*). Proteins were resolved by reducing 10% SD-PAGE, electrotransferred to nitrocellulose, and Western blotted for β-catenin. *B*, cytosolic E-cadherin and β-catenin are coprecipitated after GA treatment (*Lanes 5–8*). One mg each of 1011, 928, and 1241 melanoma cytosolic extracts were precipitated with 3 μg of anti-E-cadherin antibody, electrotransferred to nitrocellulose, and Western blotted for β-catenin. *C*, cytosolic β-catenin was precipitated from 1 mg each of 1011, 928, and 1241 melanoma cytosolic extracts, electrotransferred to nitrocellulose, and Western blotted for β-catenin (*Lanes 9–12*). *D*, total E-cadherin level was measured by Western blotting of 928 cell lysates prepared from cells exposed to 0–2 μM GA for 24 h.

The Tyrosine Kinase ErbB2 and the Tyrosine Phosphatase LAR Associate with β-Catenin in Melanoma Cells. Cells (928, 1241, and 1011) were treated with 0.5 μM GA for 12 h, lysed in Triton X-100 lysis buffer, and immunoprecipitated with antibodies specific for ErbB2, LAR, or HA (negative control). After SDS-PAGE of immunoprecipitates, immunoblots were probed for β-catenin (Fig. 2, *A–C*). Duplicate lysates were immunoblotted for ErbB2 (Fig. 2*D*), or immunoprecipitated/immunoblotted for LAR (Fig. 2*E*). In untreated cells, β-catenin was readily coimmunoprecipitated with both ErbB2 and LAR, and this was independent of β-catenin mutation or APC status. However, after GA treatment, β-catenin could no longer be coimmunoprecipitated with ErbB2, whereas its association with LAR was unaffected (Fig. 2, *A* and *B*). Because GA is known to rapidly deplete ErbB2 via stimulation of its proteasome-dependent degradation (46), we examined whether the apparent loss of association of β-catenin with ErbB2 after GA treatment was attributable to depletion of ErbB2 from the cells. As can be seen in Fig. 2*D*, GA caused essentially complete loss of ErbB2 from all three melanoma cell lines without affecting LAR protein levels (Fig. 2*E*). These results were confirmed by immunofluorescence analysis of ErbB2 and LAR proteins in 928 cells treated with GA (Fig. 3). The loss of ErbB2 after exposure of the melanoma cells to GA, without perturbation of either the LAR protein level or its association with β-catenin, is consistent with GA-induced tyrosine dephosphorylation of β-catenin, as seen in Fig. 1.

GA Inhibits β-Catenin-responsive Transcription in Melanoma Cells. Because GA-stimulated tyrosine dephosphorylation of β-catenin was correlated with its increased association with E-cadherin, we wished to determine whether β-catenin-dependent transcriptional activity, mediated by nuclear β-catenin, was also affected by GA. To examine this question, 928, 1241, and 1011 melanoma cells were first transiently transfected with either a wild-type (Topflash) or a mutated (Fopflash) Tcf-dependent luciferase reporter plasmid and constitutive β-catenin-responsive reporter activity was determined. As expected, 928 and 1241 cells, both of which harbor significant amounts of nuclear β-catenin (22, 47), display readily detectable levels of Tcf-specific reporter activity (Topflash:Fopflash ratio, 13.6 and 6.6, respectively; data not shown). On the other hand, 1011 cells, which do not express elevated levels of cytoplasmic β-catenin and contain only minimal nuclear β-catenin (47), display essentially no Tcf-specific luciferase activity (Topflash:Fopflash ratio, 1.8; data not shown). Thus, 928 and 1241 cells were selected for additional study and transiently transfected with either Topflash or the Lef-specific reporter plasmid 7LEF-fosLuc. After 24 h, cells were exposed to GA, and luciferase activity was determined. Data were normalized to cotransfected Renilla luciferase activity, which itself was unaffected by GA treatment (Fig. 4*D*). GA specifically and significantly inhibited both Topflash and 7LEF-fosLuc reporters in both 928 and 1241 cells. At the same time, β-catenin immunoblots of intact nuclei revealed a decrease in nuclear β-catenin content after GA treatment (Fig. 4*A*). To examine the effects of GA on an endogenous protein whose transcription is stimulated by β-catenin, we monitored the effects of GA on cyclin D protein levels in 928 and 1241 cells (Fig. 4*B*). Cyclin D protein disappeared from these cells, but was unaffected in 1011 cells, after GA. Thus the sensitivity of cyclin D to GA is observed only in those melanoma cells that contain transcriptionally active β-catenin. Next, we wished to determine whether the loss of cyclin D protein after GA was mediated at a pretranslational level. We monitored cyclin D-specific mRNA in 928 and 1241 cells before and after GA treatment, and we observed that the message for cyclin D completely disappeared in GA-treated cells (Fig. 4*C*). Thus, in two melanoma cell lines with constitutive β-catenin-stimulated transcription, as measured by two different reporter plasmids and the endogenous β-catenin-sensitive *cyclin D* gene, GA markedly inhibited β-catenin-driven transcription.

GA Inhibits Melanoma Cell Motility. Because the abundance of membrane-associated β-catenin-E-cadherin complexes is inversely related to cell motility (28), we wished to determine whether GA reduced the motility of 928 and 1241 melanoma cells *in vitro*. When added to a 48-h *in vitro* motility assay, GA at 0.5 μM almost completely abolished motility in both cell lines without affecting cell viability as assessed by trypan blue staining (Fig. 5). These results are consistent with the increased association of β-catenin and E-cadherin seen after GA treatment.

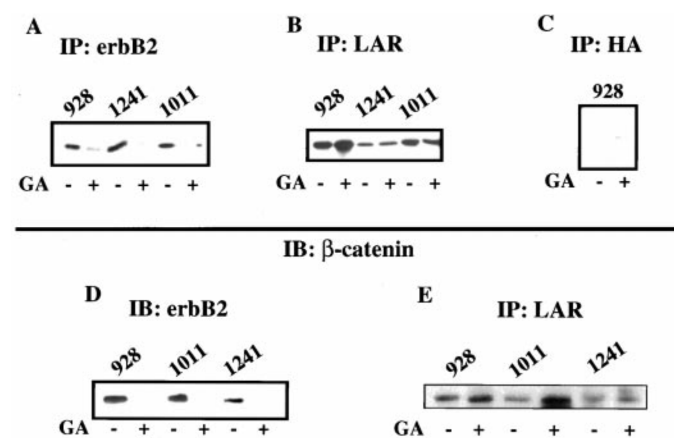

Fig. 2. To detect coimmunoprecipitation of ErbB2 and β-catenin, or LAR and β-catenin, melanoma cells were treated with GA (0.5 μM) for 12 h or left untreated. After lysis, 1 mg of total protein was immunoprecipitated with an antibody recognizing either ErbB2 (*A*) or LAR (*B* and *E*). As a negative control, lysate was also immunoprecipitated with an antibody specific for the HA peptide (*C*). Immunobeads were washed thoroughly, boiled in reducing Laemmli sample buffer, and associated proteins were resolved by 10% SDS-PAGE. After electrotransfer to nitrocellulose, the membranes were Western blotted for β-catenin (*A–C*). Before immunoprecipitation, 50 μg of total lysate proteins were resolved as above and Western blotted for ErbB2 (*D*). Duplicate samples of LAR immunoprecipitates were Western blotted for LAR (*E*).

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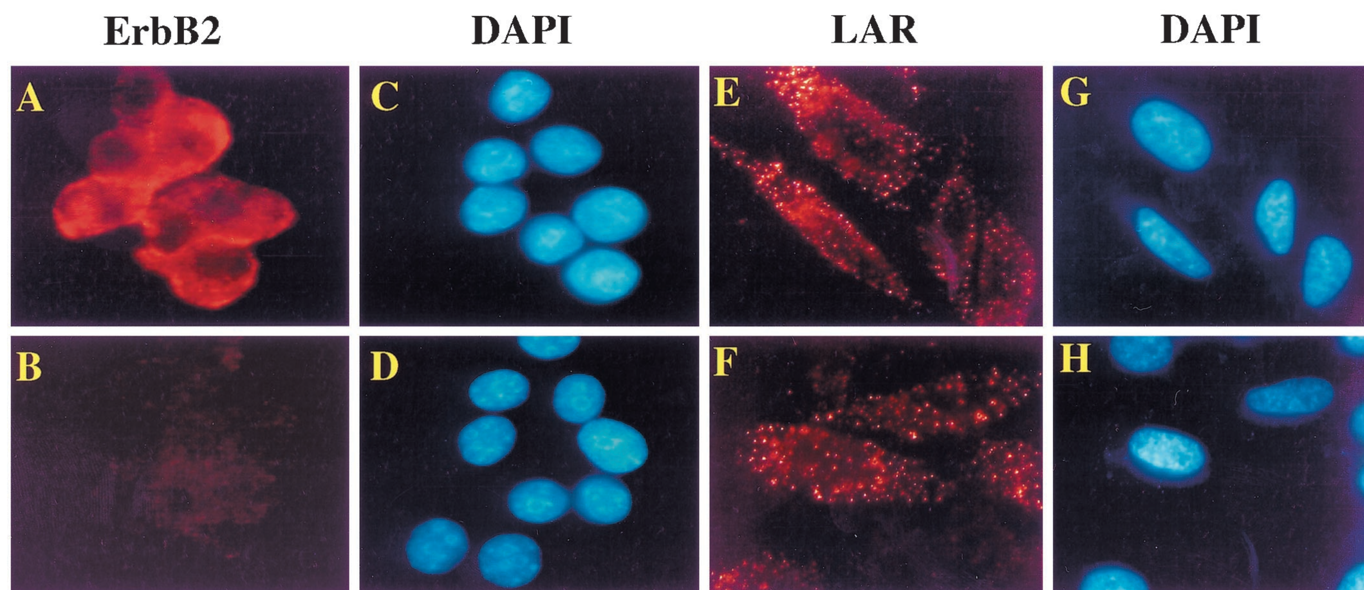

Fig. 3. Melanoma cells (928) were grown on coverslips and exposed to GA (1 μM) for 14 h or left untreated. After being rinsed in PBS, cells were fixed in 3.7% formaldehyde in PBS and permeabilized with 0.2% Triton X-100. LAR (*red*) and ErbB2 (*red*) were visualized by immunofluorescence. The DNA-intercalating dye DAPI (*blue*) was used to identify cell nuclei. *A*, *C*, *E*, and *G*, untreated cells; *B*, *D*, *F*, and *H*, GA-treated cells.

Association of β-Catenin Point Mutants Y654E and Y654F with E-Cadherin in Melanoma Cell Lines. To confirm that the enhanced β-catenin-E-cadherin association observed after GA treatment was a direct result of tyrosine dephosphorylation of β-catenin in these cells, we transiently transfected 1241 cells with HA-β-catenin harboring either a Y654F or a Y654E mutation, immunoprecipitated cell lysates with anti-HA antibody, and probed resultant immunoblots for E-cadherin (Fig. 6*A*). Endogenous E-cadherin was coimmunoprecipitated with β-catenin(Y654F) but not with β-catenin(Y654E), although the Y654E β-catenin mutant was expressed at a somewhat higher level in 1241 cells. Importantly, GA had no effect on E-cadherin association with either β-catenin point mutant, nor did GA alter the tyrosine phosphorylation of either mutant.

Transcriptional Activity of Transiently Transfected β-Catenin Point Mutants Y654E and Y654F. To examine further whether the inhibition by GA of β-catenin-stimulated transcription can be directly attributed to its effect on the tyrosine phosphorylation status of β-catenin, we transiently transfected each β-catenin point mutant into 293 cells together with either Topflash or Fopflash reporter plasmids, and we determined β-catenin-sensitive reporter activity 24 h after transfection. Both β-catenin point mutants were transcriptionally active, and, importantly, GA did not affect their activity (Fig. 6*B*). However, the transcriptional activity of β-catenin(Y654F) in 293 cells was twice as sensitive to cotransfection of E-cadherin as was the transcriptional activity of β-catenin(Y654E) (37% *versus* 76% of control activity, Fig. 6*C*).

DISCUSSION

The data in this study demonstrate that β-catenin is highly tyrosine phosphorylated in melanoma cells and minimally associated with E-cadherin irrespective of the proteasome sensitivity of β-catenin.

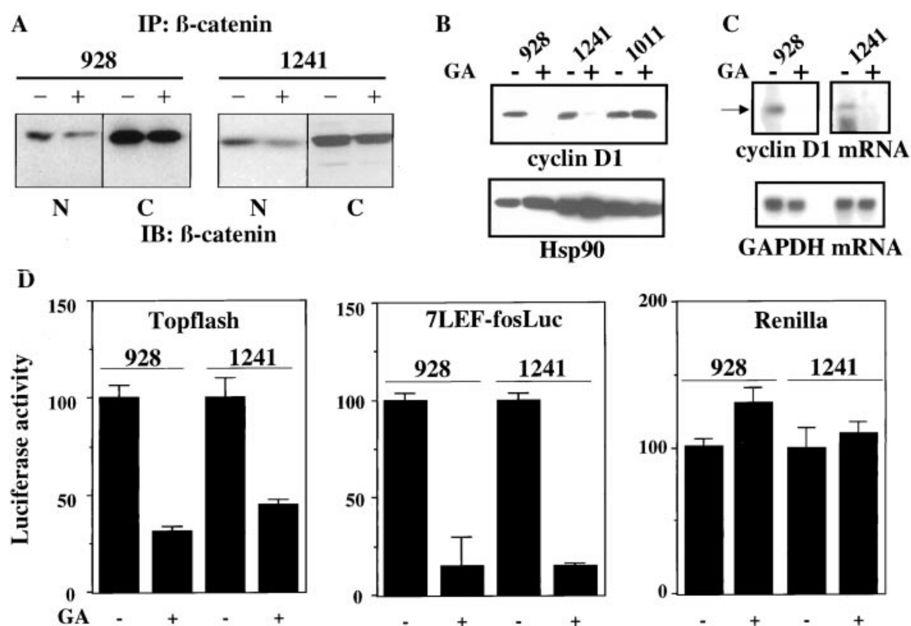

Fig. 4. Melanoma cells (928 and 1241) were treated with GA (1 μM) for 14 h or left untreated. *A*, cells were fractionated into nuclei (*N*) and cytosol (*C*); aliquots of both fractions were resolved by SDS-PAGE and immunoblotted for β-catenin. *B*, cells were lysed as in Fig. 1; duplicate aliquots of total lysate proteins were resolved by SDS-PAGE and were immunoblotted for either cyclin D1 (*B*, *top*) or Hsp90 (*B*, *bottom*). *C*, total RNA was isolated from identically treated cells, and 20 μg of total RNA were electrophoresed through a 1% agarose-formaldehyde gel, transferred to nitrocellulose, and hybridized for either cyclin D1 or glyceraldehyde-3-phosphate dehydrogenase mRNA. *D*, 928 and 1241 cells were transiently transfected with either Topflash or 7Lef-fosLuc β-catenin-sensitive reporter plasmids together with a Renilla luciferase control plasmid. After 24 h, cells were treated with GA (2 μM) for an additional 24 h; then cells were lysed and processed for measurement of luciferase activity. Data are expressed as mean ± SD of four experiments.

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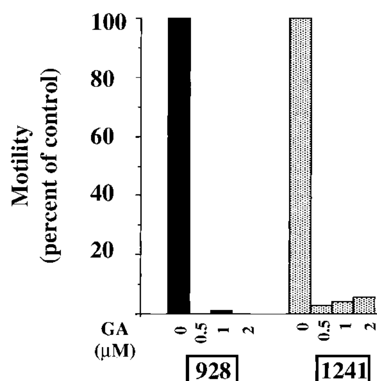

Fig. 5. Melanoma cell motility was determined as described in "Materials and Methods." GA markedly reduced the motility of 928 and 1241 melanoma cells. The data shown are representative of three independent experiments.

Although most epithelial cells must be treated with phosphatase inhibitors to visualize tyrosine phosphorylated β-catenin, our results were obtained in the absence of phosphatase inhibition, suggesting that the tyrosine phosphorylation/dephosphorylation cycle of β-catenin is unbalanced in melanoma cells. Receptor tyrosine kinase ErbB2 overexpression has been observed in the majority of melanomas examined and correlates best with metastatic melanoma (34). We found both ErbB2 and the receptor tyrosine phosphatase LAR to be coprecipitated with β-catenin in the three cell lines we studied. Thus, it is reasonable to assume that ErbB2 is responsible for the constitutive tyrosine phosphorylation of β-catenin in these melanoma cells, and that its activity overrides that of LAR, which promotes the tyrosine dephosphorylation of β-catenin *in vivo* and *in vitro* (28, 39).[3]

GA rapidly depletes ErbB2 protein from cells by stimulating its proteasome-dependent degradation, and we observed in this study that GA efficiently abrogated the tyrosine phosphorylation of β-catenin coincident with the loss of ErbB2 from the treated cells without affecting either the β-catenin steady-state level or its association with LAR. In GA-treated cells, association of tyrosine dephosphorylated β-catenin with E-cadherin was enhanced, and this occurred concurrently with dramatically reduced cell motility. Similar results have been obtained by transfecting tumor cells with LAR (28). In cells that overexpressed ectopic LAR, β-catenin tyrosine phosphorylation was abrogated and the free pool of cytoplasmic β-catenin was decreased, whereas β-catenin association with E-cadherin was increased, and cell motility in response to growth factors was markedly reduced. Our data suggest that, like overexpression of LAR, pharmacological depletion of ErbB2 shifts the balance of the tyrosine phosphorylation/dephosphorylation of β-catenin in favor of the latter. Because the Tyr-654 residue of β-catenin is contained within its ErbB2-binding domain (43), we confirmed the data obtained with GA by transiently transfecting 1241 cells with two β-catenin point mutants mimicking the phosphorylated (Y654E) and dephosphorylated (Y654F) states of Tyr-654, respectively. As predicted, endogenous E-cadherin was efficiently coprecipitated with β-catenin(Y654F) but not with β-catenin(Y654E).

Although the Tyr-86 residue of β−catenin can be phosphorylated by pp60[c-src] *in vitro*, this site appears not to be heavily phosphorylated *in vivo*, nor does its phosphorylation state influence β-catenin association with E-cadherin (30). In the current experiments, minimal tyrosine phosphorylation was observed when transiently transfected Tyr-654 point mutants were examined, suggesting that Tyr-654 is the principal β-catenin tyrosine phosphorylation site in these melanoma cell lines. However, GA did not affect residual tyrosine phosphorylation of either Tyr-654 mutant, further implicating the destabilizing effects of GA on ErbB2 in mediating the drug's inhibition of β-catenin tyrosine phosphorylation.

By disrupting contact with E-cadherin, tyrosine phosphorylation of β-catenin has been shown to lead to an increase in the free, uncomplexed cytoplasmic pool of the protein as well as to its subsequent nuclear accumulation (28). Reversal of β-catenin tyrosine phosphorylation by GA was accompanied by a reduction in both nuclear β-catenin content and β-catenin-sensitive transcriptional activity, as measured using two independent reporter constructs as well as the endogenous *cyclin D* gene. Although we cannot exclude the possibility that the effects of GA on β-catenin-sensitive transcription might be mediated by alternative mechanisms not involving β-catenin, four observations support a more direct role for β-catenin in this process. First, the transcriptional activity of cotransfected Renilla luciferase was not significantly affected by GA. Second, GA only affected cyclin D levels in the two cell lines (928 and 1241) harboring stabilized β-catenin and displaying significant β-catenin-sensitive transcriptional activity. In the third cell line (1011), GA had no effect on cyclin D (although the activity of the drug in these cells was confirmed by depletion of ErbB2). Third, GA caused the loss of cyclin D mRNA as well as of cyclin D protein in the β-catenin-overexpressing cell lines. In contrast, in cell lines which neither express aberrantly

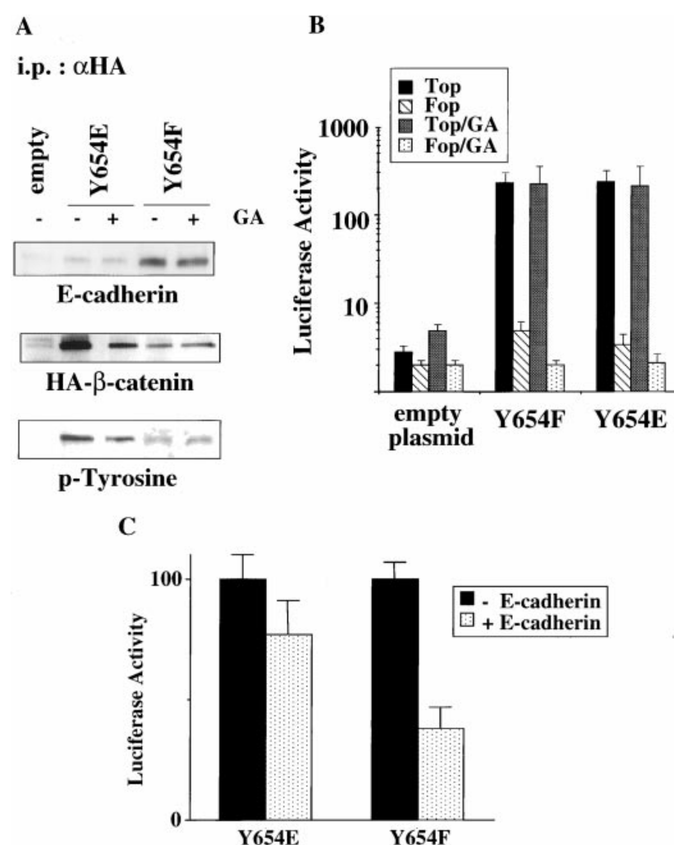

Fig. 6. *A*, 1241 cells were transfected with 1 μg of empty plasmid, β-catenin(Y654E), or β-catenin(Y654F), and, 24 h later, cells were treated with 0.5 μM GA for an additional 24 h. Cell lysates were immunoprecipitated with anti-HA antibody and probed for E-cadherin (*top*), HA-β-catenin (*middle*), or phosphotyrosine (*bottom*). *B*, 293 cells were cotransfected with 1 μg of Topflash or Fopflash, 0.5 μg Renilla luciferase, and 0.5 μg of either empty plasmid, β-catenin(Y654F), or β-catenin(Y654E). Cells were treated with GA (as in *A*), and Renilla-corrected β-catenin-dependent luciferase activity was determined. Data are expressed as mean ± SD of four experiments. *C*, 293 cells were cotransfected with 1 μg of Topflash, 0.5 μg of Renilla luciferase, 0.5 μg of E-cadherin, and 0.25 μg of β-catenin(Y654E) or β-catenin(Y654F). Renilla-corrected Topflash luciferase activity was measured 24 h after transfection. Data are expressed as mean ± SD of four experiments. ■, −E-cadherin; □, +E-cadherin.

[3] A. G. de Herreros and M. Dunach, unpublished observations.

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stabilized β-catenin nor demonstrate constitutive β-catenin-sensitive transcriptional activity, the effects of a closely related GA-derivative, herbimycin A, on cyclin D were shown conclusively to be posttranslationally mediated (48). Lastly, GA did not affect the transcriptional activity of either Tyr-654 point mutant transiently transfected into 293 cells, suggesting that ability of GA to alter β-catenin-stimulated transcription is mediated by its indirect but specific effect on the state of β-catenin tyrosine phosphorylation at residue 654.

In cells that are able to degrade efficiently uncomplexed cytoplasmic β-catenin, deregulation of β-catenin tyrosine phosphorylation may not cause an elevation in the cytoplasmic free pool of the protein, but the lack of association of β-catenin with E-cadherin in such cells would still result in enhanced motility and decreased cell-cell contacts. In support of this possibility, tyrosine phosphorylation of β-catenin has been reported to correlate with carcinoma formation and tumor invasiveness (33). In cells unable to efficiently degrade free cytoplasmic β-catenin, increased tyrosine phosphorylation of the protein should augment the level of the free pool and result in the increased nuclear accumulation of β-catenin. Thus, pharmacological abrogation of β-catenin tyrosine phosphorylation, even in cells expressing aberrantly stable β-catenin, may antagonize the tumorpromoting activity of nuclear-localized β-catenin while simultaneously enhancing the tumor suppressive activity of plasma membrane β-catenin-E-cadherin complexes. These results may be of particular relevance to the clinical utility of GA, inasmuch as a GA derivative is currently undergoing a multi-institution Phase I clinical trial in cancer patients.

ACKNOWLEDGMENTS

We thank Dr. H. Clevers for pTopflash and pFopflash Tcf luciferase reporter plasmids, Dr. R. Grosschedl for the 7Lef-fosLuc reporter plasmid, Dr. M. Streuli for anti-LAR monoclonal antibody, and Drs. M. El-Gamil and P. Robbins for the melanoma cell lines used in this study.

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