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Subcellular Localization of β-Catenin Is Regulated by Cell Density

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It is generally accepted that subcellular distribution of β-catenin regulates its function. Membrane-bound β-catenin mediates cell–cell adhesion, whereas elevation of the cytoplasmic and nuclear pool of the protein is associated with an oncogenic function. Although the role of β-catenin in transformed cells is relatively well characterized, little is known about its importance in proliferation and cell-cycle control of nontransformed epithelial cells. Using different approaches we show that in human keratinocytes (HaCaT) β-catenin is distributed throughout the cells in subconfluent, proliferating cultures. In contrast, β-catenin is nearly exclusively located at the plasma membrane in confluent, contact-inhibited cells. Hence, we demonstrate for the first time that β-catenin is translocated from the cytoplasm to the plasma membrane in response to high cell density. We conclude that β-catenin plays an important role in proliferation and mediating contact-inhibition by changing intracellular localization.

Key Words: **β-catenin; cell–cell adhesion; proliferation; contact –inhibition; keratinocytes.**

β-Catenin is the vertebrate homologue of *Drosophila* armadillo, a protein which is involved in inducing segment polarity during embryogenesis (for review see 1). In vertebrates, β-catenin is known to have two different functions: cell–cell adhesion and signal-transduction through the wnt/wingless pathway. In consistence with these distinct functions, β-catenin is localized in several intracellular pools. The membrane bound pool of β-catenin is involved in cadherin-mediated cell–cell adhesion whereas the cytoplasmic and nuclear pool are components of the wnt/wingless pathway.

In epithelial cells, E-cadherin is essential to establish and maintain cell–cell adhesion and epithelial differentiation by mediating Ca^{2+}-dependent homophilic interactions (for review see 2, 3). Intracellularly, E-cadherin is linked to the cytoskeleton by association with α-, β-, and γ-catenin (plakoglobin). Hence, one consequence of the association with β-catenin is the reduction of the amount of free cytoplasmic β-catenin (for review see 1). In addition, the cytoplasmic pool of β-catenin is controlled to remain low by proteolytic degradation. Proteolysis of β-catenin is initiated by binding to the tumor suppressor APC (adenomatous polyposis coli), GSK-3β (glycogen synthase kinase-3β) and axin/conductin (4) and finally mediated by the proteasome system (5).

Loss of E-cadherin function or impairment of the proteolytic degradation leads to an elevation of the cytoplasmic pool of β-catenin. Accumulation of cytoplasmic β-catenin is physiologically relevant, for instance, during vertebrate embryogenesis (epithelial–mesenchymal differentiation) in response to activation of the wnt-receptor and pathophysiologically relevant during oncogenesis, e.g., due to functional inactivation of APC. In both cases, β-catenin binds in the cytoplasm to transcription factors of the TCF (T cell factor)/LEF (lymphoid enhancing factor) family thereby upregulating transcription of several genes, such as mesenchymal and proliferative genes, e.g., c-myc and cyclin D1 (6, 7).

Despite this knowledge, little is known about the role of β-catenin in proliferating nontransformed epithelial cells. It has recently been shown that endogenous cytoplasmic β-catenin levels oscillate during the cell cycle suggesting that β-catenin might also play a role in regulating proliferation in normal epithelial cells (8). In addition, upregulation of the cytoplasmic pool by exogenous expression of β-catenin blocks contact-inhibition (8). We therefore hypothesized that a fine balance of cytoplasmic and membrane bound β-catenin might regulate cell proliferation and contact-dependent inhibition of growth in non-transformed epithelial cells and focused our interest on human keratinocytes, HaCaT cells. This cell line has been initially obtained from a normal skin biopsy and maintains a substantial differentiation potential in culture (9, 10) as well as contact-dependent inhibition of growth

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(present work). We studied expression and subcellular distribution of β-catenin in subconfluent, i.e., proliferating and confluent, i.e., contact-inhibited, cells. We show for the first time that β-catenin is translocated from the cytoplasm to the plasma membrane in response to high cell density. We conclude that β-catenin plays an important role in proliferation and mediating contact-inhibition by changing intracellular localization.

MATERIALS AND METHODS

Cell culture. The human keratinocyte cell line HaCaT was kindly provided by Dr. N. E. Fusenig, German Cancer Research Center, Heidelberg, and cultured in CG-medium (BioWest, France) supplemented with 0.5% fetal calf serum, penicillin and streptomycin (each 100 U/ml, Gibco) in 10% CO_2.

Determination of cell number. Cells were washed, incubated with EDTA (0.5 M)/PBS (phosphate-buffered saline), trypsinized, and counted in a hemocytometer.

[^{3}H]Thymidine incorporation assay. Cells were either sparsely seeded (10^4/well) or to confluence (7×10^4/well) in microtiter plates and cultured for 24 h. The cells were then labeled with 9.25 kBq/well of [^{3}H]thymidine for 4 h. Cells were washed with PBS, incubated with 10 μl/well of EDTA (0.5 M)/PBS for 20 min at 37°C and trypsinized by the addition of 10 μl of 2.5% trypsin for 15 min at 37°C. Cells were incubated overnight at −20°C and then harvested onto glass fiber filters. Incorporated radioactivity was determined by liquid scintillation spectrometry.

Cell extracts. HaCaT cells were either sparsely seeded (8×10^5 cells/60 mm) or seeded to confluence (3×10^6/60 mm) and cultured for 48 h. Total cell extracts were prepared by lysing the cells in hot Laemmli sample buffer (11). Separation of cytoplasmic and membrane fractions were performed according to (8) by homogenizing the cells in hypotonic buffer [10 mM Tris/HCl pH 7.4, 0.2 mM $MgCl_2$, 5 mM NEM, protease and phosphatase inhibitors as described (12)]. Cell debris were removed by centrifugation and the supernatant centrifuged for 1 h at 4°C and 100.000*g*. Proteins of the supernatant (S100) were precipitated by chloroform/methanol, the pellet (P100) dissolved in hot Laemmli sample buffer. Nuclear fractions were prepared by extraction with 10 mM Hepes/NaOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 300 mM saccharose, 1 mM EDTA, 1 mM DTT, protease and phosphatase inhibitors as described (12). The extract was resuspended by a 22-gauge needle, nuclei pelleted by centrifugation at 960*g* and finally dissolved in hot Laemmli sample buffer. Protein concentration was determined according to (13).

Western blotting. Equal amounts of protein (20–50 μg/lane) were separated by SDS–PAGE (7.5, 12.5, or 15%) and electroblotted overnight onto Immobilon membranes (Millipore). The membranes were blocked for 1 h with 5% low-fat milk powder in TBS (50 mM Tris/HCl, pH 7.5, 150 mM NaCl) containing 0.05% Tween 20 and then incubated for 1.5 h at room temperature with anti-E-cadherin- (1:2500), anti-γ-catenin- (1:1000, each Transduction Laboratories), anti-β-catenin-, anti-α-catenin-, and anti-pRB-antibodies (each 1:1000, Santa Cruz). Secondary antibodies coupled to horseradish peroxidase were added for additional 1 h followed by ECL-detection according to the manufacturer's instruction (Cell Signaling, UK). Equal loading was controlled by Coomassie blue staining (data not shown). Quantification was carried out by NIH Image.

Immunofluorescence. HaCaT cells were either sparsely seeded (8×10^5 cells/60 mm) or seeded to confluence (3×10^6/60 mm) on glass coverslips and cultured for 48 h. Cells were fixed with ice-cold acetone for 2 min. The coverslips were air-dried and washed for three times with PBS. Unspecific binding was suppressed by blocking for 30 min with 5% bovine serum albumin/PBS at room temperature. The cells were then incubated with anti-β-catenin antibody (1:200, Santa Cruz) for 90 min at room temperature in a humidified chamber. After three washing steps, incubation with Cy3-conjugated anti-rabbit antibody (1:800, Jackson Immunoresearch) for 90 min at room temperature was performed. Control cells were incubated only with the secondary antibody. The cells were again washed and mounted on glass slides in a medium consisting of 17% Mowiol 4-88 (Hoechst) and 20% glycerin in 0.15% Tris/HCl pH 8.5. The cells were viewed on a Microphot-FXA Nikon microscope with an excitation wavelength of 550 nm.

RESULTS AND DISCUSSION

HaCaT Cells Show Contact-Dependent Inhibition of Growth

We first investigated if HaCaT cells which are known to be immortalized due to a mutation in the p53 gene (14) are subjected to contact-dependent inhibition of growth. Cells were seeded to 50% confluence and cultured for 3 days. Figure 1A shows that the cell number did not significantly increase when the cells had reached the critical cell density of about 7.5×10^6 cells/cm^2. When HaCaT cells were seeded to confluence and cultured for 24 h DNA synthesis decreased to 20% compared to subconfluent, proliferating cells (Fig. 1B). These data revealed that HaCaT cells are regulated by contact-dependent inhibition of growth.

Since contact-inhibited cells are arrested in G1-phase, we next investigated phosphorylation of the retinoblastoma gene product (pRB). pRB encodes a 105- to 114-kDa nuclear phosphoprotein, which is tightly bound to E2F (and other transcription factors) and histone deacetylase in its hypophosphorylated state thereby inhibiting gene transcription of S-phase specific genes (15, 16). (Hyper)phosphorylation leads to its functional inactivation resulting in the loss of binding to E2F, thus permitting progression into S-phase (for review see 17). Since hyperphosphorylation of pRB results in a marked electrophoretic shift (15), it can be easily detected by Western blotting. Figure 1C clearly demonstrates that pRB is present in its hyperphosphorylated state in proliferating cells. In contrast, we could only detect the hypophosphorylated, growth-inhibitory species of pRB in confluent HaCaT cells which is consistent with G1 arrest.

pRB is phosphorylated during G1 progression by the cyclin D(1, 2, 3)/cdk4 complex and downstream by the cyclin E/cdk2 complex (for review see 18). In consistence with pRB being hypophosphorylated in confluent cultures we detected a downregulation of the cdk-activating subunits cyclin D1 and D3 (D2 could not be detected in HaCat cells) and an increase in the cdk4-inhibitor p15 as well as the cdk2-inhibitor p27 in confluent HaCaT cells (data not shown). These data are in accordance with the results in other cell lines (19, 20, and manuscript submitted).

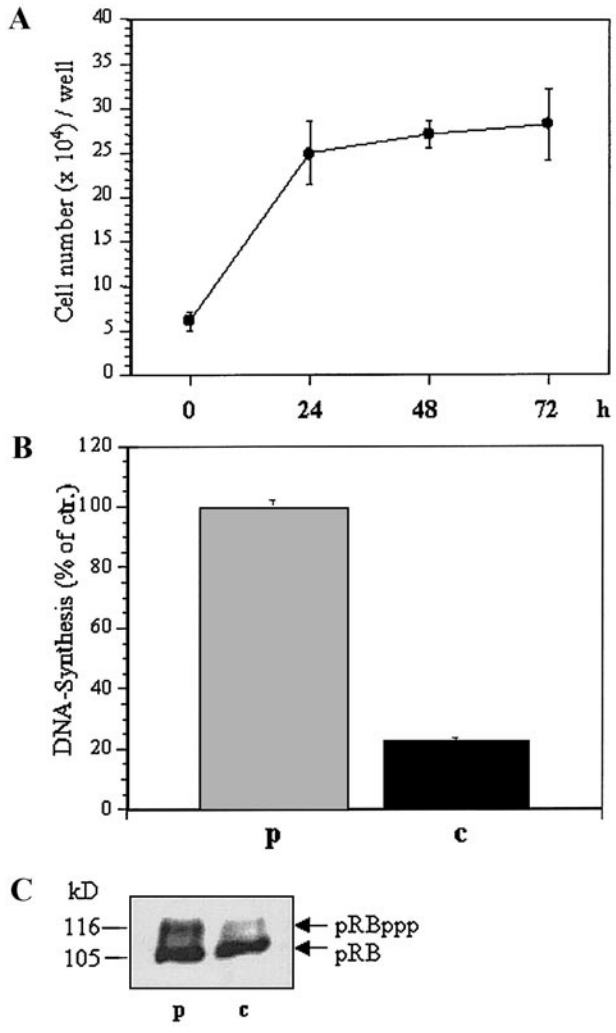

FIG. 1. HaCaT cells show contact-dependent inhibition of growth. (A) HaCaT cells were seeded to 50% confluence and cultured for 72 h. Cell number was determined at the indicated time points. Results are given as $x \pm s_x$ ($n = 4$). (B) HaCaT cells were either sparsely seeded (proliferating, p) or seeded to confluence (c). DNA synthesis was measured after 24 h by the incorporation of [^{3}H]thymidine. Results are given as $x \pm s_x$ ($n = 4-6$). (C) HaCaT cells were either sparsely seeded (p, proliferating) or to confluence (c) and cultured for 48 h. Equal amounts of protein were subjected to Western blotting with anti-pRB-antibody. Coomassie blue staining was performed to control equal loading (data not shown). One representative blot out of three independent experiments leading to similar results is shown.

Expression of E-Cadherin, β-, and γ-Catenin Is Increased in Confluent HaCaT Cells

E-cadherin has been shown to be the major cell adhesion molecule in epithelial cells. However, it is not known whether E-cadherin is involved in contact-dependent inhibition of growth. We therefore examined the expression of E-cadherin as well as of the intracellularly associated proteins α-, β-, and γ-catenin in subconfluent, i.e., proliferating and confluent, i.e., contact-inhibited HaCaT cells. Western blot analysis revealed an approximately 9-fold elevation of E-cadherin protein levels in confluent cultures (Fig. 2). In addition, the protein levels of β-, and γ-catenin were increased about 4- and 2.5-fold, respectively, in response to high cell density. The protein levels of α-catenin were not altered in confluent HaCaT cells. These data indicate that protein levels of E-cadherin, β-, and γ-catenin are regulated by cell density and suggest an involvement of the E-cadherin/catenin complex in contact-dependent inhibition of growth.

β-Catenin Is Translocated to the Plasma Membrane in Response to High Cell Density

To get more insight into the role of β-catenin we studied subcellular distribution of the protein in subconfluent and confluent HaCaT cells. Immunofluorescence revealed that β-catenin was located at the plasma membrane in subconfluent cells, but also to a significant amount distributed throughout the cells (Fig. 3A). In contrast, β-catenin was exclusively located at the plasma membrane in confluent cells. No staining could be observed in the cytoplasm or nucleus (Fig. 3A). Since γ-catenin was located at the plasma membrane in subconfluent and in confluent HaCaT cells (data not shown) we conclude that the change in subcellular distribution of β-catenin was a specific response to high cell density. A similar translocation of β-catenin from the cytoplasm to the plasma membrane was observed in rat liver epithelial cells (manuscript in preparation) indicating that our observation was not restricted to a single cell line.

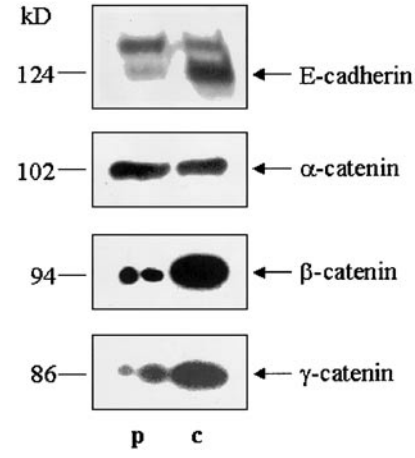

FIG. 2. Protein levels of E-cadherin, β-, and γ-catenin are upregulated in confluent cultures. HaCaT cells were seeded and cultured as described in the legend to Fig. 1C. Equal amounts of total cell extracts were subjected to Western blotting using anti-E-cadherin-, anti-α-, anti-β-, or anti-γ-catenin antibodies. Equal loading was controlled by Coomassie blue staining (data not shown). The molecular weight is shown on the left. Representative blots are shown out of two independent experiments leading to similar results.

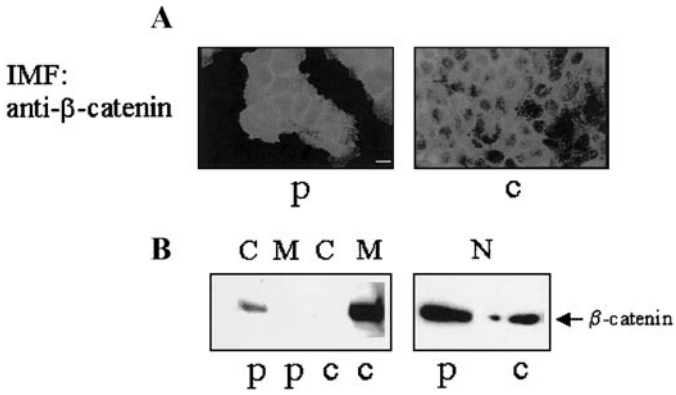

FIG. 3. Subcellular distribution of β-catenin depends on cell density. (A) HaCaT cells were seeded and cultured as described in the legend to Fig. 1C. Indirect immunofluorescence was performed with anti-β-catenin-antibody and Cy3-conjugated secondary antibody. The bar represents 20 μm. (B) HaCaT cells were seeded and cultured as described in Fig. 1C. Subcellular fractionation into cytoplasmic (C), membrane (M) and nuclear (N) fractions was performed as described under Materials and Methods. Western blot analysis was performed using anti-β-catenin-antibodies. Equal loading was controlled by Coomassie blue staining (data not shown). One representative blot out of three independent experiments leading to similar results is shown.

To support our finding of a redistribution of β-catenin in response to cell-density subcellular fractionation and subsequent Western blotting was performed. Cell extracts were either separated into a cytoplasmic and membrane fraction or nuclei were isolated. Figure 3B clearly shows that β-catenin is found in the cytoplasmic and nuclear fraction in subconfluent cells, but predominantly detected in the membrane fraction in confluent cultures. Hence, our data demonstrate for the first time that β-catenin is translocated from the cytoplasm and nucleus to the plasma membrane in response to high cell density.

It is generally accepted that subcellular distribution of β-catenin determines its function. The membrane pool is associated with E-cadherin hence mediating cell adhesion, the cytoplasmic pool may be involved in cell proliferation by binding to transcription factors of the TCF/LEF family thereby regulating transcription of proliferative genes, such as c-myc and cyclin D1 (6, 7). In nontransformed cells, the cytoplasmic pool is kept to be low by proteolytic degradation (4, 5). However, our data show that a substantial amount of cytoplasmic and nuclear β-catenin is present in proliferating HaCaT cells. This indicates that even low amounts of cytoplasmic β-catenin might be important for cell proliferation in nontransformed cells.

The fact that β-catenin is translocated to the plasma membrane in confluent cells further suggests a role in contact inhibition. It is known that E-cadherin and APC compete for the interaction with β-catenin (21). Interestingly, subcellular localization of APC is also regulated by cell density (22). Our findings suggest that cell proliferation is regulated by sequestering free cytoplasmic β-catenin to the plasma membrane in confluent HaCaT cells thereby reducing the amount of free cytoplasmic β-catenin. Hence, the observed up-regulation of E-cadherin in confluent cultures exceeding that of β-catenin provides a reasonable explanation for the subcellular redistribution of β-catenin to the plasma membrane in contact-inhibited cells.

In summary, our data reveal for the first time a translocation of β-catenin from the cytoplasm to the nucleus in response to high cell –density, indicating that β-catenin is involved in cell proliferation and contact-dependent inhibition of growth in nontransformed HaCaT cells.

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