Differential Regulation of Endogenous Cadherin Expression in Madin-Darby Canine Kidney Cells by Cell-Cell Adhesion and Activation of β-Catenin Signaling*

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Cadherins mediate cell-cell adhesion, but little is known about how their expression is regulated. In Madin-Darby canine kidney (MDCK) cells, the cadherin-associated cytoplasmic proteins α- and β-catenin form high molecular weight protein complexes with two glycoproteins (Stewart, D. B., and Nelson, W. J. (1997) J. Biol. Chem. 272, 29652–29662), one of which is E-cadherin and the other we show here is the type II cadherin, cadherin-6 (K-cadherin). In low density, motile MDCK cells, the steady-state level of cadherin-6 is low, but protein is synthesized. However, following cell-cell adhesion, cadherin-6 becomes stabilized and accumulates by >50-fold at cell-cell contacts while the E-cadherin level increases only 5-fold during the same period. To investigate a role of β-catenin in regulation of cadherin expression in MDCK cells, we examined the effects of expressing signaling-active β-catenin mutants (ΔGSK, ΔN90, and ΔN131). In these cells, while levels of E-cadherin, α- and β-catenin are similar to those in control cells, levels of cadherin-6 are significantly reduced due to rapid degradation of newly synthesized protein. Additionally, these cells appeared more motile and less cohesive, as expression of ΔGSK-β-catenin delayed the establishment of tight confluent cell monolayers compared with control cells. These results indicate that the level of cadherin-6, but not that of E-cadherin, is strictly regulated post-translationally in response to Wnt signaling, and that E-cadherin and cadherin-6 may contribute different properties to cell-cell adhesion and the epithelial phenotype.

Cadherins comprise a family of widely expressed, transmembrane glycoproteins that mediate the initiation, specificity, and maintenance of cell-cell adhesion (2, 3). During embryonic development, complex patterns of cadherin expression are thought to facilitate changes in cell migration and morphology, influence cell, fate and contribute to the establishment of tissue architecture and function (4–6). Misexpression of cadherins has been correlated with altered adhesion phenotypes of numerous cancers, and it has been proposed that E-cadherin can function as a tumor suppressor gene (7–9).

The extracellular domain of cadherins mediates calcium-dependent, homotypic binding between opposed cells, and the intracellular domain binds cytoplasmic proteins, termed catenins (10). β-Catenin binds directly to the cytoplasmic domain of cadherin and to α-catenin, an actin-binding protein (11). This hierarchy of protein interactions connects the cadherin-catenin complex to the actin cytoskeleton, thereby leading to the clustering and stabilization of the complex at the plasma membrane (12, 13). β-Catenin binding is also required for efficient transport of cadherin to the plasma membrane (14).

In addition to its role in cell adhesion, β-catenin is a homolog of the Drosophila segment polarity protein armadillo and a central player in the Wnt/Wg signaling pathway (15, 16). Activation of this pathway results in inhibition of Zw-3/GSK-3β kinase, leading to the accumulation of hypophosphorylated β-catenin/armadillo (15, 17). This signaling-active form of β-catenin is in a low molecular weight pool and is not complexed with E-cadherin (18, 19). Accumulation of β-catenin in this pool is thought to drive it into a complex with members of the TCF/Lef-1 family of transcription factors in the nucleus where the complex directly activates target gene transcription (20, 21). In the absence of Wnt signaling, GSK-3β is active and β-catenin is phosphorylated, which leads to rapid ubiquitination and degradation of cytosolic β-catenin by the 26 S proteasome (19, 22, 23). Deletions (ΔN90, ΔN131) or amino acid substitutions (ΔGSK) of putative N-terminal phosphorylation sites for GSK-3β in β-catenin result in a signaling-active form of β-catenin that can induce TCF-mediated gene transcription (19).

Although many studies have examined the effects of cadherin overexpression, misexpression, and inhibition on cellular organization, little is known about mechanisms involved in the regulation of endogenous cadherin expression and function (24, 25). The involvement of β-catenin in both cell-cell adhesion and a signaling pathway raises the possibility that these two pathways are connected. Although it has been reported that overexpression of Wnt-1 in AtT20 cells correlates with increased levels of β-catenin and N-cadherin and with increased calcium-

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1 The abbreviations used are: GSK, glycogen synthase kinase; MDCK, Madin-Darby canine kidney; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; ALLN, N-acetyl-Leu-Leu-norleucinal; TCF, T cell factor; kb, kilobase(s); DMEM, Dulbecco’s modified Eagle’s medium; Dox, doxycycline; PVDF, polyvinylidene difluoride; RT, reverse transcription; PCR, polymerase chain reaction; HRP, horseradish peroxidase; TBS, Tris-buffered saline; HSB, high stringency buffer; CSK, cytoskeleton extraction buffer.
dependent cell-cell adhesion (26), a similar study in PC12 cells revealed an apparent decrease in cell-cell adhesion (27). Additionally, the promoter for the E-cadherin gene has an upstream consensus sequence for TCF binding which could be a target for β-catenin/TCF regulation (21). However, studies with mutant forms of E-cadherin and armadillo in Drosophila indicate that the adhesion and signaling properties of β-catenin are separate (28, 29). We sought to further define members of cadherin- and catenin-containing complexes in Madin-Darby canine kidney (MDCK) cells and to examine changes in these complexes in the presence and absence of signaling-active forms of β-catenin. We have identified cadherin-6 as a second, major cadherin in MDCK cells. Significantly, we demonstrate differential regulation of E-cadherin and cadherin-6 expression and function in these cells, both after initiation of cell-cell contact and in response to the expression of β-catenin mutants, which activate the Wnt-1/Wg signaling pathway.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Antiserum—**Madin-Darby canine kidney type II/J (MDCK J) and MDCK type II/G (MDCK G) cells have been described previously (30). MDCK clone T23 (MDCK T23), ΔN90, ΔN131, and ΔGSK cell lines have also been described previously (19, 31). MDCK T23, ΔN90, ΔN131, and ΔGSK cells were maintained in DMEM supplemented with 10% FBS and 100 μg/ml each of penicillin, streptomycin, and amphotericin B. Mouse monoclonal antibodies specific for β-catenin, E-cadherin, and desmoglein-1 were purchased from Transduction Laboratories (Lexington, KY). Polyclonal antibodies specific for cadherin-6 (SpK3) were raised in rabbits against the peptide sequence PHVEPRFLYLGPFKDSC, coupled against the peptide sequence PHVEPRFLYLGPFKDSC, coupled through the cysteine to maleimide activated keyhole limpet hemocyanin (Pierce).

**Metabolic Labeling—**For chase and pulse-chase metabolic labeling experiments, 2.5 × 10⁶ cells were seeded on 24-mm collagen-coated Transwell filters (Corning Incorporated, Corning, NY) and grown for 3 days. When labeling to steady-state, cells on filters were washed three times with labeling medium (DMEM minus methionine/cysteine [Met/Cys]) incubated for 22 h in overnight labeling medium (labeling medium with 10% DMEM [v/v]) with 0.4 μCi of [35S]Met/Cys added to the basolateral side of each Transwell filter. 0.4 μCi of additional radioactive label was added to the basolateral side after 11 h of incubation. Cells were then either harvested immediately (0 h chase) or washed three times with chase medium (DMEM supplemented with 10,000-fold excess unlabelled Met/Cys), incubated for 1 or 6 h, and then extracted in 1.1 ml of CSK buffer (50 mM NaCl, 30 mM sucrose, 10 mM PIPES pH 6.8, 3 mM MgCl₂, 0.5% Triton X-100) containing protease and phosphatase inhibitors (0.1 mM Na₃VO₄, 50 mM NaF, 1 mM Pefabloc (Roche Molecular Biochemicals), and 10 μg/ml each of leupeptin, antipain, aprotinin, chymostatin, and pepstatin A). Extracts were centrifuged 20 min at 22,000 × g, and when indicated, the remaining pellet (CSK insoluble) was solubilized by boiling in 100 μl of 1% SDS buffer (10 mM Tris, pH 7.5, 1% SDS [w/v], 1 mM EDTA) and then diluted with 1 ml of CSK buffer prior to processing for immunoprecipitation. For determination of molecular ratios, the number of methionines and cysteines per protein were estimated based on sequences submitted to GenBank® (mutant cadherin-6), the full-length cadherin-6 cDNA or a BamHI/BglII fragment with the complete mouse E-cadherin cDNA, 12; rat E-cadherin, 16. For pulse-chase experiments, cells on filters were washed two times with labeling medium, preincubated for 30 min in labeling medium, and pulse-labeled for 15 min at 37°C in labeling medium + 250 μCi of [35S]Met/Cys per filter. After the labeling period, cells were chased for times indicated and harvested as described above for chase samples.

**RNA Extraction—**MDCK cells were plated at a density of 5 × 10⁵ cells/100-mm culture dish, and total RNA was extracted 2 days after plating with the RNeasy Total RNA Kit as described by the manufacturer (Qiagen, Valencia, CA).

**RT-PCR and Cloning of a MDCK Cadherin-6 cDNA Fragment—**RT-PCR was performed with the 5’ primer 223GGAAGTTACCATCAGACCGAGTAGAGGAGGATCATG250 and the 3’ primer 1010TCTCTGTATTGCTCTCTTGGATTGATGCAAAGGCTC1927 using total RNA from T23 MDCK cells cultured without Dox and the Access RT-PCR kit as described by the manufacturer (Promega, Madison, WI). The 1.7-kb PCR product was cloned into the pBluescript II SK+ vector (Stratagene, La Jolla, CA). The identity of the cloned cDNA, encoding a region corresponding to amino acids 75–653 in human cadherin-6, was confirmed by sequencing. This sequence region encoded a peptide with 95% sequence identity to mouse cadherin-6 (GenBank® accession no. D28209), 97% identity to rat cadherin-6 (GenBank® accession no. D25290) and 98% identity to human cadherin-6 (GenBank® accession no. L40035). For comparison with the homology between human and rat cadherin-6 is 97% for the mature protein. A 1.5-kb SacI fragment of the MDCK cadherin-6 cDNA or a BamHI/Xhol fragment with the complete human α-tubulin cDNA (kindly provided by Dr. Eugenio de Hostos, University of California, San Francisco) was labeled with [α-32P]dCTP using the high prime DNA labeling kit as described by the manufacturer (Roche Molecular Biochemicals). The labeled cDNAs were separated from unincorporated nucleotides with a Sephadex G-50 DNA column (Nick Column; Amersham Pharmacia Biotech) and used as a probe for hybridization to Northern blots of total RNA from the different MDCK cell lines.

**Northern Blotting—**Agarose gel electrophoresis, Northern blotting onto Hybond N nylon membrane, and hybridization were performed as described by the manufacturer of the nylon membrane (Amersham Pharmacia Biotech).

**Cell Surface Biotinylation—**Cells were seeded and grown on 24-mm Transwell filters as described above. Cells were biotinylated at 4°C with 20 μg/ml non-reducible biotin (NH4-biotin; Pierce), washed five times with ice-cold Tris-buffere saline buffer (TBS; 20 mM Tris-HCl, pH 7.5, 150 mM NaCl), three times with Heps-buffere saline (20 mM Hepes, pH 7.5, 150 mM NaCl), extracted in DHE buffer with inhibitors, and pulselabeled as described previously (18). To clear glycoproteins from samples, extracts were incubated with either concanavalin A-Sepharose 4B (Sigma) or with Sepharose 4B as a control. For detection of biotinylated proteins, membranes were blocked overnight in TBS + 7.5% ovalbumin, washed once with TBS, twice with TBS + 0.1% Tween 20, incubated with an HRP-avidin (Vectorstain ABC kit; Vector Laboratories, Burlingame, CA), washed six times with TBS + 0.1% Tween 20,
then visualized with enhanced chemiluminescence reagent (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

RESULTS

MDCK Cells Express Cadherin-6—In previous studies, we identified two high molecular weight complexes containing α- and β-catenin in MDCK cells (1). One of these complexes contained E-cadherin, but the components of the other complex were not defined. In order to identify proteins in this second high molecular weight complex, we used a β-catenin antibody to immunoprecipitate protein complexes from [35S]Met/Cys-labeled MDCK cell extracts that had been fractionated by Superose 6 gel FPLC (Fig. 1A). The β-catenin immunoprecipitates from fractions containing high molecular weight catenin complexes (fractions 15–20) had four proteins, three of which could be identified by their apparent molecular masses and reactivity to specific antibodies: proteins of 120, 102, and 95 kDa, corresponding to E-cadherin, α-catenin, and β-catenin, respectively. A protein of 130 kDa did not react with any of these antibodies (Fig. 1A, arrowhead). Note that the 120-kDa protein (E-cadherin) eluted in a peak at fraction 16, whereas the unknown 130-kDa protein first appeared in a peak at fraction 17, α-Catenin and β-catenin co-eluted in a peak between fractions 16 and 17.

To confirm that the 130-kDa protein was not E-cadherin, we first immune-depleted [35S]Met/Cys-labeled MDCK cell extracts of E-cadherin by six consecutive rounds of immunoprecipitations with an anti-E-cadherin antibody. Note that, in addition to E-cadherin, these antibodies recognize several classical cadherins, including P- and N-cadherin (11). Following immune depletion, extracts were fractionated by Superose 6 FPLC. β-Catenin immunoprecipitates from the E-cadherin immune-depleted lysate prior to FPLC fractionation (Fig. 1B, −EC) and following Superose 6 FPLC fractionation (Fig. 1B, fractions 15–20) contained three major proteins, the unidentified 130-kDa protein and α-catenin and β-catenin, all of which co-eluted as a complex in a peak at fraction 17. As expected, E-cadherin was not present in any of these column fractions.

To begin the characterization of the 130-kDa protein, we examined whether it was a cell surface glycoprotein (Fig. 1, C–E). Cells grown on Transwell filters were biotinylated for 20 min at 4 °C with a non-reducible biotin (sulfo-NHS-biotin), and subsequently extracted with DHE buffer (see “Experimental Procedures” for details). Extracts were incubated with concanavalin A-Sepharose 4B (Fig. 1, C–E, lanes 2 and 4) to bind glycosylated proteins, or with Sepharose 4B beads (Fig. 1, C–E, lanes 1 and 3) as a control. Following centrifugation to remove the beads, the remaining extract was immunoprecipitated with polyclonal antibodies against either E-cadherin (Fig. 1, C–E, lanes 1 and 2) or β-catenin (Fig. 1, C–E, lanes 3 and 4). Using avidin-HRP to detect biotinylated proteins, E-cadherin immunoprecipitates contained a 120-kDa protein (Fig. 1C, lane 1), and the β-catenin immunoprecipitate contained 120- and 130-kDa cell surface proteins (Fig. 1C, lane 3). Neither the 120- nor 130-kDa biotinylated protein was detected in immunoprecipitates from extracts pre-cleared with concanavalin A-Sepharose 4B (Fig. 1C, lanes 2 and 4), indicating that they were both glycoproteins. As predicted, E-cadherin and β-catenin were
detected in E-cadherin and β-catenin immunoprecipitates from extracts pre-cleared with Sepharose 4B beads (Fig. 1, D and E, lanes 1 and 3). Additionally, neither E-cadherin nor β-catenin was detected in the E-cadherin immunoprecipitates from concanavalin A-Sepharose 4B-treated extracts (Fig. 1, D and E, lane 2), and only a small amount of β-catenin remained in β-catenin immunoprecipitates from concanavalin A-Sepharose 4B-treated extracts (Fig. 1, D and E, lane 4).

Additional β-catenin immunoprecipitations were performed on extracts from cells grown on filters and biotinylated as above. Extracts were either untreated (Fig. 1, C–E, +E-cad, lane 5) or were immune-depleted of E-cadherin (Fig. 1, C–E, –E-cad, lane 6) prior to immunoprecipitation. Again, avidin-HRP revealed the presence of 120- and 130-kDa biotinylated proteins co-immunoprecipitated with β-catenin from untreated samples (Fig. 1C, lane 5), and a 130-kDa biotinylated protein in β-catenin immunoprecipitations from E-cadherin immune-depleted extracts (Fig. 1C, lane 6). No E-cadherin, and only a reduced level of β-catenin, was detected in the β-catenin immunoprecipitate from the extract that had been immune-depleted of E-cadherin (Fig. 1, D and E, lane 6) relative to the untreated control β-catenin immunoprecipitation (Fig. 1, D and E, lane 5). Together, these data indicate that p130 is a glycosylated, cell surface protein that binds β-catenin and is not recognized by a polyclonal antibody raised against the cytoplasmic domain of E-cadherin.

In order to identify p130, we performed large scale β-catenin immunoprecipitations of this protein complex to obtain sufficient amounts of p130 for peptide microsequencing. Sequence analysis of three peptides from the 130-kDa protein revealed an exact match with corresponding sequences in rat K-cadherin and human cadherin-6 (Fig. 1F).

Cadherin-6 and E-cadherin Form Distinct Complexes with Catenins in MDCK Cells—To analyze cadherin-6 expression in MDCK cells, we generated a polyclonal antibody (SpK3) against a peptide (PHVEPRFLYLGPFDSC) whose sequence is found in the extracellular domain of cadherin-6, but not in E-cadherin or other known catenins. In Fig. 2A, total protein from a 1% SDS extract of MDCK T23 cells was processed for immunoblotting with the anti-cadherin-6 antibody. In Fig. 2B, equal amounts of total protein from three independent lines of MDCK cells were separated by 7.5% SDS-PAGE, transferred to PVDF membrane, and probed for immunoblotting with antibodies specific for cadherin-6, E-cadherin, and β-catenin. The antibody generated against the cadherin-6 peptide sequence recognized a single 130-kDa protein in all three cell lines.

To further test the specificity of the antibodies, SpK3 (cadherin-6) and 3G8 (E-cadherin) antibodies were used to immunoprecipitate and immunoblot cadherin-6 and E-cadherin complexes from MDCK cell CSK extracts (for details, see “Experimental Procedures”). The E-cadherin immunoprecipitate contained E-cadherin but not cadherin-6 (Fig. 2C, lane 1), while the cadherin-6 immunoprecipitate contained cadherin-6 but not E-cadherin (Fig. 2C, lane 2). The level of cadherin-6 in CSK extracts from MDCK cells was not reduced following immune depletion of E-cadherin, while the level of β-catenin was reduced by approximately 60%, consistent with the removal of one of β-catenin’s binding partners from the extract (Fig. 2D, lanes 1 and 2). β-Catenin immunoprecipitates from similar CSK extracts contained both cadherin-6 and E-cadherin (Fig. 2D, lane 3), while extracts immune depleted of E-cadherin removed all of the fraction of β-catenin associated with E-cadherin and left the level of cadherin-6 unaltered (Fig. 2D, lanes 3 and 4). These data indicate strongly that E-cadherin and cadherin-6 are in separate complexes containing β-catenin.

**Fig. 2. Cadherin-6 and E-cadherin form distinct complexes with β-catenin.** A, a 1% SDS extract was prepared from MDCK T23 cells as described under “Experimental Procedures.” The extract was separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with an antibody specific for cadherin-6 (C6). This antibody recognized a single major protein of 130 kDa. B, 1% SDS extracts from three strains of MDCK cells (T23, type IIA, and type IIG) were prepared as described above and processed for immunoblotting with antibodies specific for cadherin-6, E-cadherin, and β-catenin. C, MDCK cells were extracted with CSK buffer and processed for immunoprecipitation with antibodies specific for E-cadherin (lane 1) or cadherin-6 (lane 2). Complexes were separated by SDS-PAGE, and antibodies specific for E-cadherin or cadherin-6 were used to detect these proteins in the immunoprecipitates (lanes 1 and 2) and in the extracts prior to immunoprecipitation (lanes 3 and 4). D, CSK buffer MDCK cell extracts were untreated (lanes 1 and 3) or E-cadherin immunodepleted (lanes 2 and 4). Samples were either separated directly by SDS-PAGE (lanes 1 and 2) or processed for immunoprecipitation with antibodies specific for β-catenin prior to separation (lanes 3 and 4). Electrophoretically separated proteins were transferred to PVDF membrane and immunoblotted with antibodies specific for cadherin-6, E-cadherin, or β-catenin. E, MDCK cells were labeled with [35S]Met/Cys to steady-state, extracted with 1% Triton X-100, and processed for immunoprecipitation with antibodies specific for E-cadherin (EC), cadherin-6 (C6), or β-catenin (BC). Immunoprecipitated complexes were stringently washed and then separated in 7.5% acrylamide gels. Arrows denote relative electrophoretic mobilities of indicated catenins and catenins. In A and E, bars on the left denote the relative electrophoretic mobilities of molecular weight markers: myosin (M_r = 205,000), β-galactosidase (M_r = 116,000), phosphorylase b (M_r = 97,400), bovine serum albumin (M_r = 68,000), and ovalbumin (M_r = 45,000).

We next sought to determine the relative amounts of E-cadherin and cadherin-6-associated with β-catenin. MDCK cells were grown on Transwell filters, labeled with [35S]Met/Cys for 20 h, and then extracted with CSK buffer. Extracts were divided into three equal portions and subjected to immunoprecipitation with antibodies specific for cadherin-6, E-cadherin, or β-catenin (Fig. 2E). The cadherin-6 immunoprecipitate (Fig. 2E, C6) contained major proteins of 130, 102, and 95 kDa that correspond to cadherin-6, α-catenin, and β-catenin, respectively. E-cadherin immunoprecipitates (EC) contained 120-kDa E-cadherin, and α- and β-catenin. Proteins in the β-catenin immunoprecipitate (BC) were identified as cadherin-6, E-cadherin and α- and β-catenin. Quantification of the relative amounts of cadherin-6 and E-cadherin in the β-catenin immunoprecipitate, when corrected for differences in methionine and cysteine contents, revealed E-cadherin (120 kDa) and cadherin-6 (130 kDa) in an ~3:2 ratio (Fig. 2E).

In order to confirm that cadherin-6 is part of a high molecular weight protein complex with catenins, MDCK cells were
extracted with DHE buffer and fractionated by Superose 6 FPLC (Fig. 3). Immunoblot analysis showed that cadherin-6 eluted in a single, high molecular weight peak at fraction 17. E-cadherin eluted in a single high molecular weight peak with a peak at fraction 16. Significantly, the distributions of cadherin-6 and E-cadherin overlapped with that of the high molecular weight pool of catenins in fractions 15–19. In summary, these data demonstrate that cadherin-6 is present at a level similar to that of E-cadherin in confluent monolayers of MDCK cells, that it is present in a high molecular weight complex with catenins, and that the cadherin-6/catenin and E-cadherin/catenin complexes are distinct.

**Cadherin-6 and E-cadherin Accumulate at Steady State with Different Kinetics following Initiation of Cell-Cell Contact**—Since cadherin-6 and E-cadherin form separate protein complexes, we next sought to investigate the possibility that cadherin-6 and E-cadherin are regulated independently by examining protein levels in cells at different times after induction at cell-cell contacts. Results show that the level of E-cadherin had increased by ~5-fold and reached a steady-state level within 24 h after initiation of cell-cell contact (Fig. 4, A and B). In contrast, the level of cadherin-6 increased significantly over the time course such that at 96 h after induction of cell-cell adhesion its level was ~50-fold greater than that at 0 h. These data demonstrate that cadherin-6 and E-cadherin have different rates of accumulation following initiation of cell-cell contact, and indicate that the expression of cadherin-6 and E-cadherin are regulated independently.

**Expression of Mutant Forms of β-Catenin Decreases the Steady-state Cadherin-6 Level by Increasing Protein Turnover**—Previously, we identified changes in cadherin and catenin complexes as a result of cellular transformation and expression of β-catenin mutants (19). Because of differences in the regulation of E-cadherin and cadherin-6 in MDCK cells (see above), we sought to examine cadherin-6 expression in MDCK cell lines expressing mutant forms of β-catenin. These cell lines have been described and characterized previously, and express mutant β-catenin lacking N-terminal 90 (ΔN90) or 131 (ΔN131) amino acids or having amino acid substitutions S33A, S36A, T40A, and S44A in the N-terminal consensus phosphorylation sites for GSK-3β kinase (19, 31). Expression of these mutant β-catenins in T23 MDCK cells is under the control of the tetracycline/doxycycline-repressible transactivator (see Ref. 31).

In parental T23 MDCK cells, levels of cadherin-6, E-cadherin, α-catenin, β-catenin, plakoglobin, and desmoglein-1 (a desmosomal cadherin) were the same in both the presence or absence of doxycycline (Fig. 5). However, upon expression of ΔGSK-, ΔN90-, or ΔN131-β-catenin, the level of cadherin-6 was dramatically reduced. With prolonged exposure of the cadherin-6 immunoblot, a series of lower molecular weight proteins are visible in the samples from ΔGSK-, ΔN90-, and ΔN131-β-catenin expressing cells indicating that there was accelerated degradation of cadherin-6 in these cells. Note that the levels of E-cadherin, desmoglein-1, α-catenin, and plakoglobin in cells expressing mutant forms of β-catenin (−Dox) were similar to those in control (+Dox) cells (Fig. 5).

β-Catenin has been shown to be a regulator of gene transcription through its interaction with the Lef-1/TCF family of transcription factors (35–37). Therefore, we tested whether expression of mutant forms of β-catenin, all of which induce TCF-activated transcription in MDCK cells (19), affected cadherin-6 transcription. Northern blots for cadherin-6 mRNA revealed two transcripts of ~4.6 and 3.9 kb (Fig. 6A). Previous studies have also reported two major cadherin-6 transcripts of similar sizes in cells (38, 39). The blots were quantitated using a PhosphorImager and then re-probed with a tubulin cDNA probe as a control for RNA loading and transfer efficiency. The blot shown is representative of two independent experiments.
expressing ΔGSK, ΔN90-, and ΔN131-β-catenin. Parental (T23)
MDCK cells ± Dox and MDCK cells expressing ΔN90-, ΔN131-, and
ΔGSK-β-catenin ± Dox were seeded on plastic tissue culture plates
and grown for 3 days. Cells were extracted with 1% SDS, separated
by SDS-PAGE, transferred to PVDF membranes, and immunoblotted
with antibodies specific for desmoglein-1 (DG-1), cadherin-6, E-cadherin,
α-catenin, β-catenin, or plakoglobin. Equal amounts of total protein
were loaded in each lane. The presence of a triplet of proteins detected
with the DG-1 antibody reflects different glycosylated forms of the protein.

(Fig. 6A), and the data are plotted as the average ± S.D. of
these experiments (Fig. 6B). These data reveal that the levels
of the two major cadherin-6 mRNA transcripts are not signifi-
cantly different between control cells and cells expressing
ΔGSK-, ΔN90-, or ΔN131-β-catenin.

Since there was not a change in the level of cadherin-6
mRNA, we next tested whether the change in cadherin-6 pro-
tein level in cells expressing mutant forms of β-catenin was due
to a difference in the rate of cadherin-6 protein turnover. We
examined this question in cells expressing the ΔGSK mutant of
β-catenin since this mutant should not disrupt interactions
between cadherin, catenins, and the actin cytoskeleton (19). In
control cells (+Dox), the rates of cadherin-6 and E-cadherin
degradation were similar (t½ = 5.5 ± 2.9 h for cadherin-6, and
~6.5 ± 1.7 h for E-cadherin) (Fig. 7A). In contrast, while
expression of ΔGSK-β-catenin (~Dox) had little effect on E-
cadherin turnover (t½ = 5.5 ± 2.6 h), the rate of cadherin-6
turnover was greatly accelerated (t½ = 0.87 ± 0.48 h).

Because the level of cadherin-6 present in cells expressing
ΔGSK-β-catenin was very low following steady-state labeling with [35S]Met/Cys, we examined the maturation of newly
synthesized cadherin-6 after a short pulse-labeling period. Cad-
herin-6 immunoprecipitates from control cells (+Dox) revealed
that cadherin-6 was synthesized as a 115-kDa precursor (p),
which was processed to its final 130-kDa mature (m) form
between 30 and 60 min after synthesis (Fig. 7B). In the same
cells, E-cadherin was synthesized as a 115-kDa precursor and
was processed within 30–60 min to its final 120-kDa form
(Fig. 7D). In cells expressing ΔGSK-β-catenin, cadherin-6
appeared to be synthesized at a rate similar to that in control
cells (+Dox), but the majority of the 115-kDa cadherin-6 precursor
did not chase into the 130-kDa mature form (Fig. 7D). Instead,
most of the cadherin-6 precursor was rapidly degraded within
60 min following synthesis. Note that in cells expressing
ΔGSK-β-catenin (~Dox), E-cadherin is synthesized and proc-
essed at rates similar to those in control cells (+Dox).

ΔGSK-β-Catenin Expression Changes the Intracellular Dis-
bution of Cadherin-6 and Alters Cell and Colony Morphol-
ologies—We examined the subcellular localization of cadherin-6 in
ΔGSK-β-catenin-expressing cells by indirect immunofluores-
cence microscopy. In control cells (ΔGSK, +Dox), cadherin-6
was localized to sites of cell-cell contact, which overlapped with
the distributions E-cadherin and β-catenin (Fig. 8). Consistent
with previous studies (19), expression of ΔGSK-β-catenin re-
sulted in an elevated level of intracellular β-catenin while
E-cadherin localization remained unchanged. Significantly, in
ΔGSK-β-catenin-expressing cells (ΔGSK, ~Dox), cadherin-6
staining revealed a diffuse intracellular pattern, and plasma
membrane localization was reduced compared with that in
control cells. A similar staining pattern for cadherin-6 was also
present in cells expressing ΔN90- or ΔN131-β-catenin. Note
that cadherin-6 staining in ΔGSK-β-catenin cells does not nec-
essarily indicate the presence of full-length protein since the
antibody used was generated against a peptide from the third
extracellular repeat region.

To assess whether the reduction in cadherin-6 levels corre-
lates with changes in cell and colony morphology, contact-naive
ΔGSK-β-catenin cells were seeded at single-cell density on
plastic dishes and photographed using a phase-contrast micro-
scope at 5, 16, 25, and 40 h after plating. Shortly after plating

FIG. 5. Steady-state cadherin-6 protein levels in MDCK cells
expressing ΔGSK, ΔN90-, and ΔN131-β-catenin. A, total RNA from
 parental (T23), ΔGSK, ΔN90, and ΔN131 cells grown ± Dox was sepa-
rated in 1% agarose gels and transferred to Hybond NX nylon mem-
brane. Membranes were processed for Northern blotting, and cad-
herin-6 and tubulin mRNA were detected using appropriate
[35S]dCTP-labeled cDNA probes. B, intensities of radioactive signals
from hybridized cDNA probes in A were quantitated using a Phos-
phorImager, and values shown are averages of two experiments ± 1 S.D.
Cadherin-6 signals were normalized against the corresponding tubulin
signal, and resulting values were plotted relative to the value obtained
for T23-Dox cells. Cadherin-6 transcripts of 4.6 and 3.9 kb were quan-
titated and plotted independently.

FIG. 6. Comparison of cadherin-6 mRNA levels in MDCK cells
expressing ΔGSK-, ΔN90-, and ΔN131-β-catenin. A, total RNA from
parental (T23), ΔGSK, ΔN90, and ΔN131 cells grown ± Dox was sepa-
rated in 1% agarose gels and transferred to Hybond NX nylon mem-
brane. Membranes were processed for Northern blotting, and cad-
herin-6 and tubulin mRNA were detected using appropriate
[35S]dCTP-labeled cDNA probes. B, intensities of radioactive signals
from hybridized cDNA probes in A were quantitated using a Phos-
phorImager, and values shown are averages of two experiments ± 1 S.D.
Cadherin-6 signals were normalized against the corresponding tubulin
signal, and resulting values were plotted relative to the value obtained
for T23-Dox cells. Cadherin-6 transcripts of 4.6 and 3.9 kb were quan-
titated and plotted independently.
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Fig. 7. Cadherin-6 but not E-cadherin is rapidly degraded in MDCK cells expressing ΔGSK-β-catenin. A, ΔGSK cells ± Dox were labeled to steady-state with [35S]Met/Cys and then chased for 0, 1, or 6 h in the presence of excess unlabeled Met/Cys. Cells were extracted with CSK buffer (S), centrifuged, and the remaining pellet (P) was solubilized with 1% SDS. Supernatant and pellet were processed for immunoprecipitation with antibodies specific for cadherin-6 or E-cadherin. Immunoprecipitated complexes were washed stringently, separated by SDS-PAGE, and detected by fluorography. B–E, ΔGSK cells grown either with (B and D) or without Dox (C and E) were pulse-labeled for 15 min with 250 μCi of [35S]Met/Cys and then chased for 0, 5, 15, 30, 60, or 180 min in the presence of excess unlabeled Met/Cys. At the end of each chase period, cells were extracted with CSK buffer, extracts were divided, and protein was immunoprecipitated with antibodies against cadherin-6 (B and C) and E-cadherin (D and E). Protein complexes were washed stringently, separated by SDS-PAGE, and detected by fluorography. The letters "p" and "m" indicate the electrophoretic mobilities of the precursor (p) and mature (m) forms of cadherin-6 and E-cadherin. Bars on the left of the autoradiographs (B–E) indicate relative electrophoretic mobilities of molecular weight markers: β-galactosidase (M₆ = 116,000) and phosphorylase b (M₉ = 97,400).

and prior to colony formation, control cells (+Dox) and ΔGSK-β-catenin cells (−Dox) had similar morphologies of spindly, fibroblast-like outlines (Fig. 9). However, by 16 h, control cells had begun to form small, compact colonies with smooth rounded edges, while ΔGSK-β-catenin cells continued to appear flattened and fibroblastic. As cell density increased, ΔGSK-β-catenin cells began to form loosely packed colonies with irregular edges. ΔGSK-β-catenin colonies were markedly flatter and less compacted than control colonies, although they eventually formed confluent monolayers. Note that previous studies have shown that cells expressing ΔN90- or ΔN131-β-catenin have cell and colony morphology changes similar to those seen in ΔGSK-β-catenin cells (31).

Inhibitors of the 26 S Proteasome and Lysosomes Do Not Block Cadherin-6 Degradation—β-Catenin, and other components of the Wnt signaling pathway, have been shown to be rapidly degraded by the 26 S proteasome (22, 40). Since cadherin-6 is also degraded in cells expressing an activated β-catenin-signaling pathway, we used N-acetyl-Leu-Leu-norleucinal (ALLN), a general inhibitor of neutral cysteine proteases and the proteasome, to attempt to block cadherin-6 degradation.

Parental T23 MDCK cells (±Dox) and cells expressing ΔGSK-β-catenin (±Dox) were grown in the presence of 20 μM ALLN for 12–14 h. Immunoblots of whole cell extracts show that ALLN treatment was effective in causing the accumulation of higher molecular weight forms of β-catenin, but it did not prevent the degradation of cadherin-6. In order to determine if lysosomes were involved in cadherin-6 degradation, we incubated cells expressing ΔGSK-β-catenin (±Dox) for 4–6 h in the presence of 250 μM chloroquine, or 10 mM ammonium chloride to block lysosomal degradation. No effect was observed on cadherin-6 levels under these conditions. We conclude that neither proteosomal nor lysosomal inhibitors increased steady-state levels of cadherin-6 in these cells.

**DISCUSSION**

Previous studies of cell adhesion complexes in MDCK cells showed that E-cadherin was the major cadherin in MDCK cells, and that N- and P-cadherin were expressed in small amounts (41). We identified two high molecular weight cadherin-catenin complexes in MDCK cells based on a slight, but reproducible difference in the fractionation profiles of E-cadherin and α/β-catenin following Superose 6 FPLC. One complex that contained α/β-catenin reacted with an antibody specific to E-cadherin and a "pan-cadherin" antibody raised against the cytoplasmic domain of E-cadherin, whereas the second complex contained a 130-kDa cell surface glycoprotein that did not react with either antibody. Microsequencing of three different peptides obtained from purified 130-kDa protein revealed 100% identity to cadherin-6 (K-cadherin).

Through our analyses of MDCK cell cadherins, we have identified significant differences in the regulation of E-cadherin and cadherin-6 expression. First, during the development of confluent monolayers of polarized MDCK cells, we found that the steady-state cadherin profile shifts from being predominantly E-cadherin in contact-naive cells to a final E-cadherin: cadherin-6 ratio of −3:2 in polarized cells. At present, the functional consequences of different ratios of E-cadherin and cadherin-6 are unclear. However, given the fact that E-cadherin reaches maximum steady-state levels between 5–10 h following initiation of cell-cell contacts, at a time when the cadherin-6 level is still very low, we suggest that E-cadherin plays the central role in establishing initial cell-cell contacts and early stages in generating cell polarity. Since the amount of cadherin-6 accumulates gradually following cell-cell adhesion, it may play a role in maintaining and stabilizing cell-cell contacts that had been initiated by E-cadherin, and it is possible that a contact-derived signal is necessary to initiate cadherin-6 accumulation. The nature of this signal is at present unknown.

The second difference in the regulation of E-cadherin and cadherin-6 was our finding that the level of cadherin-6, but not E-cadherin is regulated in the presence of mutant forms of β-catenin that mimic activation of the Wnt signaling pathway (42, 43). We found that expression of mutant β-catenin specifically reduced the steady-state amount of cadherin-6 to a low level similar to that found in contact-naive MDCK cells, while at the same time not affecting the level of E-cadherin. This low level of cadherin-6 in cells expressing mutant β-catenin was independent of cell confluence or time after induction of cell-cell contacts.

Since it is known that β-catenin-TCF complexes activate transcription of several genes, including those for c-Myc, cyclin D1, c-Jun, and Fra-1 (44–47), it was possible that the decrease in cadherin-6 levels in cells expressing a signaling-active form

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2 The data regarding the effects of proteasome and lysosomal inhibitors on cadherin-6 degradation are available as a figure upon request.
of β-catenin was initiated at the transcriptional level. However, this was not the case as the levels of cadherin-6 mRNA transcripts were unchanged when expression of mutant β-catenin was repressed (+Dox) or not (−Dox). Instead, we found that the cadherin-6 level was regulated at a post-translational stage. Metabolic pulse-chase experiments revealed a decrease in the t½ of the 130-kDa cadherin-6 from 6 h to 1 h following induction of ΔGSK-β-catenin expression. The t½ of E-cadherin was 6 h in both the presence or absence of ΔGSK-β-catenin (see also Ref. 32). The molecular basis for the rapid degradation of cadherin-6 in cells expressing ΔGSK-β-catenin is unclear. Although inhibitors of both the 26 S proteasome and lysosomal proteases were ineffective at blocking cadherin-6 degradation, we note that degradation of mutant E-cadherin and misfolded cystic fibrosis transmembrane regulator are slowed, but not completely inhibited by proteasome inhibitors, indicating that other proteolytic mechanisms are involved in the degradation of these proteins (14, 48). Alternatively, β-catenin signaling may result in up-regulation of a protease that may have specificity for cadherin-6 as well as other unidentified targets. Accordingly, recent studies have shown that β-catenin may play a role in the regulation of matrilysin transcription (49); however, it is unknown whether matrilysin interacts with cadherin-6. Up-regulation of a specific protease may result in intracellular degradation and/or extracellular cleavage of cadherin-6 from the cell surface.

In addition, our data showed that newly synthesized cadherin-6 is processed from a premature 115-kDa form into a mature 130-kDa form approximately 30–60 min after synthesis. Interestingly, the time course of cadherin-6 processing is very similar to the time course of E-cadherin processing, which occurs during E-cadherin transport to the cell surface (13). However, in cells expressing signaling-active β-catenin (−Dox) resulted in intracellular accumulation of both β-catenin and cadherin-6.

**Fig. 8. Intracellular accumulation of cadherin-6 in ΔGSK-β-catenin cells.** 2 × 10⁵ cells were seeded on cover-slips, grown for 2.5 days, fixed with 4% paraformaldehyde, permeabilized with CSK buffer, and double-stained with antibodies specific for cadherin-6 and E-cadherin or cadherin-6 and β-catenin. Polyclonal cadherin-6 antisera were detected with rhodamine-conjugated goat anti-rabbit secondary antibodies, and monoclonal E-cadherin and β-catenin antibodies were detected with fluorescein-conjugated goat anti-mouse secondary antibodies. Note significant overlap of cadherin-6 staining with E-cadherin and β-catenin staining at regions of cell-cell contact in control (+Dox) cells. Expression of activated β-catenin (−Dox) resulted in intracellular accumulation of both β-catenin and cadherin-6. Bar = 20 μm.
are expressed transiently during neural and renal development (52, 53). Cadherin-6 mRNA is expressed in restricted patterns in fetal rat kidney and brain, and selective cadherin expression may play a role in the establishment of early neural segmentation and tubule differentiation (39, 54, 55). Activation of Wnt signaling could be important in these processes, not only for regulating gene transcription, but also in post-translational control of cadherins (26). In MDCK cells expressing an signaling-active form of β-catenin, loss of cadherin-6-mediated adhesion does not appear to affect the initiation of cell-cell contacts, nor the formation of an epithelial monolayer. However, the cells are noticeably more flat and form less compact colonies than control cells (Fig. 9; see also Ref. 31). Thus, the response of an individual cell to Wnt may depend on the repertoire of cadherins that are expressed.

In the developing kidney, it is possible that Wnt signaling affects cadherin expression at both the transcriptional and post-translational levels, thereby influencing cell association and cell-fate determination. Expression of Wnt-11 at the ureteric bud tip correlates with E-cadherin expression in the ureteric bud and in the portions of the mesenchyme-derived renal vesicle, comma-shaped body, and S-shaped body nearest to the bud tip. In contrast, the region of the renal vesicle distal to the ureteric bud tip expresses cadherin-6, and this region will eventually form the straight segments of the proximal tubule in the adult (56). Differential regulation of E-cadherin and cadherin-6 by Wnt expression could give rise to specific adhesion patterns between cells and altered morphogenetic development (56–58). In this context it is interesting to note that under conditions in which the level of cadherin-6 is low (contact-naïve cells, or cells expressing a signaling-active form of β-catenin), MDCK cells tend to be more spindly and fibroblast in shape, and more migratory. In contrast, an increased level of cadherin-6 upon cell-cell adhesion corresponds to a change in cell shape to a columnar form and a decrease in migratory activity. Recently, expression of N-cadherin has been shown to correlate with increased migration of cells even in the presence of E-cadherin-mediated cell-cell adhesion (59). Additionally, Si80 cells transected with cadherin-7 have been shown to disperse more rapidly than N-cadherin-transfected cells, both when plated on fibronectin-coated substrates and when grafted into neural crest cell migratory pathways of chicken embryos (44–47). Together, these results suggest that combinatorial expression of cadherins may affect not only cell-cell adhesion but also, independently, cell migration and morphology. Therefore, the differential regulation of specific cadherin subtypes may be an additional mechanism by which Wnt and other morphogenetic pathways determine cell fate.

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