Cadherin function in junctional complex rearrangement and posttranslational control of cadherin expression

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Troxell, Megan L., Yih-Tai Chen, Nicole Cobb, W. James Nelson, and James A. Marrs. Cadherin function in junctional complex rearrangement and posttranslational control of cadherin expression. Am. J. Physiol. 276 (Cell Physiol. 45): C404–C418, 1999.—The role of E-cadherin, a calcium-dependent adhesion protein, in organizing and maintaining epithelial junctions was examined in detail by expressing a fusion protein (GP2-Cad1) composed of the extracellular domain of a nonadherent glycoprotein (GP2) and the transmembrane and cytoplasmic domains of E-cadherin. All studies shown were also replicated using an analogous cell line that expresses a mutant cadherin construct (T151) under the control of tet repressor. Mutant cadherin was expressed at ~10% of the endogenous E-cadherin level and had no apparent effect on tight junction function or on distributions of adherens junction, tight junction, or desmosomal marker-proteins in established Madin-Darby canine kidney cell monolayers. However, GP2-Cad1 accelerated the disassembly of epithelial junctional complexes and delayed their reassembly in calcium switch experiments. Inducing expression of GP2-Cad1 to levels approximately threefold greater than endogenous E-cadherin expression levels in control cells resulted in a decrease in endogenous E-cadherin levels. This was due in part to increased protein turnover, indicating a cellular mechanism for sensing and controlling E-cadherin levels. Cadherin association with catenins is necessary for strong cadherin-mediated cell-cell adhesion. In cells expressing low levels of GP2-Cad1, protein levels and stoichiometry of the endogenous cadherin-catenin complex were unaffected. Thus effects of GP2-Cad1 on epithelial junctional complex assembly and stability were not due to competition with endogenous E-cadherin for catenin binding. Rather, we suggest that GP2-Cad1 interferes with the packing of endogenous cadherin-catenin complexes into higher-order structures in junctional complexes that results in junction destabilization.

E-cadherin; adherens junction; tight junction

EPITHELIAL CELL-TO-CELL junctional complexes include tight junctions (zonula occludens; ZO), adherens junctions (zonula adherens), and desmosomes (macula adherens) (11). These junctions maintain structural integrity and physiological function of epithelial tissues and are a locus of intercellular signaling machinery. Adherens junctions, tight junctions, and desmosomes share a common structural organization; all three types of junctional complexes consist of transmembrane components with adhesive function and cytosolic adaptor proteins that provide a link to the cytoskeleton. In the case of adherens junctions, catenins link the transmembrane E-cadherin protein to the actin-based cytoskeleton (27, 50, 55; reviewed in 42, 63, 65). Tight junctions are also linked to the actin cytoskeleton; the transmembrane protein occludin binds directly to ZO-1, which binds actin and ZO-2 (16, 17, 21, 30, 62; reviewed in 5, 10, 64). Desmosomal cadherins, distantly related to E-cadherin, are linked to intermediate filaments through cytosolic proteins of the desmosomal plaque, including desmoplakin and plakoglobin (reviewed in 9, 20).

During junctional complex assembly, cadherin-catenin complexes are stabilized at sites of cell-cell contact (2, 4, 37, 58) and subsequently develop into mature adherens junctions. The cadherin superfamily of adhesion molecules shares common structural features: an extracellular domain with calcium-binding sequence repeat motifs, a single transmembrane domain, and a COOH-terminal cytoplasmic domain (39, 56; reviewed in 63). Classical cadherins (e.g., E-, P- and N-cadherin) bind homotypically to identical family members on neighboring cells (27, 47; reviewed in 63). The conserved cytoplasmic domain interacts with the cytoskeleton, leading to strengthened cell-cell adhesion (40, 49, 50; reviewed in 42, 63, 65). Specifically, cadherin interactions with catenins (α-catenin, 102 kDa; β-catenin, 97 kDa; γ-catenin, also called plakoglobin, 86 kDa) have been extensively characterized (1, 12, 25, 31, 36, 41, 49; reviewed in 42, 44). Cadherin-catenin complexes also mediate intracellular signaling (reviewed in 6, 38, 54). Coordinate assembly of junctional complexes is dependent on E-cadherin (22, 23, 52, 53). Evidence placing E-cadherin at the top of the hierarchy of epithelial junctional complex assembly has come largely from experiments in which E-cadherin function was completely inhibited. Incubating epithelial cells in low-calcium medium (LCM), or in medium containing E-cadherin function-blocking antibodies, prevents assembly of adherens junctions, tight junctions, and desmosomes (22, 23, 52, 53, 59). Furthermore, cells genetically lacking α-catenin also lack E-cadherin function, and junctional complexes do not assemble (67). Several studies have employed mutant cadherins with a truncated extracellular domain to study perturbations of cadherin-based adhesion (3, 14, 32, 69). Formation of both adherens junctions and desmosomes was inhibited in cells overexpressing mutant cadherin molecules (3, 14, 69). How these mutant cadherin molecules generate an abnormal phenotype is unclear. Some investigators have suggested that the phenotype
is due to competition between mutant cadherin and endogenous cadherin for catenin binding, resulting in a decrease in catenin association with endogenous cadherin that compromises cadherin-catenin complex function (32, 69).

Here, we analyze the effects of mutant E-cadherin fusion proteins on the assembly and disassembly of cell-cell junctional complexes. Our results show that the mutant cadherin, when expressed at very low levels (10% that of endogenous E-cadherin), allowed assembly of functionally and morphologically normal junctions, but junctional complex stability and assembly rates were affected. Stoichiometry of mutant cadherin and endogenous cadherin and direct analysis of cadherin-catenin complexes in cells expressing low levels of mutant cadherin showed that competition for catenins was not responsible for the observed phenotype. We also found that mutant cadherin overexpression in Madin-Darby canine kidney (MDCK) cells reduced steady-state endogenous E-cadherin levels. Together, these results provide new insight into roles of E-cadherin in junctional complex organization and how mutant cadherin strategies affect cell-cell adhesion.

MATERIALS AND METHODS

Cell culture and transfection. MDCK cells (type II, strain G; Ref. 35) and transfected cell lines were maintained in DMEM (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Hydclone, Logan, UT), penicillin, streptomycin, and glutamine (GIBCO-BRL), and cells were passaged by trypsinization.

MDCK IIG cells were cotransfected with a fusion protein (GP2-Cad1), composed of the extracellular domain of a non-adherent glycoprotein (GP2) and the transmembrane and cytoplasmic domains of E-cadherin, and with pSV2neo (60) using calcium phosphate (19). G418 (GIBCO-BRL, 0.4 mg/ml) was used to select for transfected clones. Drug-resistant colonies were isolated and were screened for GP2-Cad1 expression by immunofluorescence and immunoblotting using 4A9, an anti-GP2 monoclonal antibody (see below). More than 10 clones were isolated that all showed virtually identical immunofluorescence staining patterns and immunoblot profiles. Low-passage-number cells were used in all experiments (passage 15 or less). Two clones were extensively characterized. MDCK cells transfected with wild-type GP2 have been described previously (35).

MDCK T23 cells were cotransfected with pUC1–1 [which encodes glutathione S-transferase-E-cadherin (GST-ECad) fusion protein; see below] and pCB7 (which confers hygromycin resistance; Ref. 7) or cotransfected with pDOX151 (which encodes T151 mutant cadherin) and pCB7 using lipofectamine, according to the recommended protocol (GIBCO-BRL). Cells were selected using hygromycin, and positive clones were identified by immunoblotting with an anti-cadherin cytoplasmic domain polyclonal antibody (see below).

For experiments, GP2-Cad1 and control cells were plated at a density of 1.5 million per filter on six-well polycarbonate filter inserts (Corning-Costar, Kennebunk, ME) and were used 8 days after plating. Sodium butyrate was utilized to select nontransfected cells and was added to the apical and basal-lateral culture media 48 h before harvest; control medium and sodium butyrate-containing medium were replaced once ~24 h before harvest. Doxycycline treatment was performed by diluting 20 µg/ml stock solution in culture medium to 0.1 or 20 ng/ml concentration. Cells were maintained in 20 ng/ml doxycycline to prevent phenotypic drift. For experiments, T151 cells were cultured in the different doxycycline concentration for 5 days before experiments were performed.

Computer graphics and image analysis. Computer graphics and image analysis were performed using Adobe Photoshop and ImagePro Plus (Media Cybernetics). Continuous data are presented as mean ± SE. ANOVA was performed where appropriate, and 

T151 mutant cadherin construct was derived from mouse E-cadherin cDNA (pSUM, a gift of Rolf Kenler, MPI Freiberg; Ref. 61). This plasmid was digested with AatII. Two AatII internal fragments were deleted, removing 1,170 base pairs (390 amino acids) of the cadherin extracellular domain. The remaining E-cadherin sequences were religated. A HA epitope tag was inserted into the deleted cadherin plasmid by digesting with EcoRI, filling in with Klenow, and then ligating a 54-base pair HA sequence (derived from plasmid pSKHAV2, a gift of Drs. Lisa Elferink and Richard Scheller). The tagged cadherin mutant, T151, was directionally cloned into pBO1/KpnI sites of the eukaryotic expression vector pCB6+. To express T151 behind a tetracycline-repressible promoter, the entire T151 sequence was amplified with the following primers: 5′pcB6+ + Xba (5′ CTC GTC TAG AGA ACC GCT 3′) and 3′pcB6+ (5′ GCC GAG GCA CTG GGG 3′). The PCR product was digested with XbaI and ligated to an XbaI-digested pCB6+ vector. This plasmid was named pDOX151.

GST-ECad fusion protein was subcloned from a bacterial expression plasmid into a eukaryotic expression vector. PCR was used to amplify a previously described fusion protein consisting of GST and the cytoplasmic domain of mouse E-cadherin (34). The following oligonucleotides were used for PCR: 5′GEXH3 (5′ TCG CAT TAT GAG CTT TAA TGT GAG CGG 3′) and 3′GEXXba (5′ GGC CGA TCT AGA TGC TCA GTG CTC AG). The resulting PCR product was ligated into the HindIII/XbaI sites of pCB6+ (a gift of Dr. Vikas Sukhatme; Ref. 7) downstream of the CMV promoter, and named pUC1–1.

Antibodies and reagents. GP2 monovalent antisera, 4A9, was a kind gift from Dr. Anson Lowe and has been described previously (13). HA monovalent antibody 12CA5 was pur-
chased from BABCO (Berkeley, CA). The rr-1 hybridoma, recognizing the extracellular domain of E-cadherin (22), and the ZO-1 hybridoma (R26.4C; Ref. 62) were purchased from Developmental Studies Hybridoma Bank maintained by the Department of Biological Sciences, The University of Iowa, Iowa City, IA, under contract from the National Institute of Child Health and Human Development. Conditioned ascites fluid was used for rr-1 immunoprecipitation experiments, and conditioned culture medium was used for rr-1 immunoblotting experiments. Conditioned culture medium was used for all experiments using the ZO-1 monoclonal antibody. Decma-1 ascites, which also recognizes the E-cadherin extracellular domain (66), was purchased from Sigma (St. Louis, MO). An anti-occludin polyclonal antibody was purchased from Zymed (South San Francisco, CA). Antiserum to the cadherin cytoplasmic domain (ECAD-B.5) was raised in rabbits against a GST-E-Cad cytoplasmic domain fusion as antigen and has been characterized (34). Anti-β-catenin and anti-α-catenin polyclonal antisera were gifts from Dr. Inke Náthke and have been described previously (26). The desmoplakin polyclonal rabbit antiserum was described in Pasdar and Nelson (52).

Chemicals were purchased from Sigma or Midwest Scientific (St. Louis, MO) unless otherwise noted.

For calcium replacement experiments, 8-day filter-grown cells were washed once in LCM (DMEM with 5 µM CaCl₂; Refs. 46, 51) and incubated with LCM plus 2.5% dFBS (FBS extensively dialyzed against Tris-saline; see Refs. 46, 51). At time points from 15 to 240 min after calcium removal, cells were washed and fixed immediately for immunofluorescence staining (see below).

For calcium replacement experiments, 8-day filter-grown cells were washed in LCM and switched to LCM plus 2.5% dFBS as above for 2, 3, or 4 h. LCM was then replaced with complete DMEM [normal (high)-calcium medium (HCM) containing 1.8 mM CaCl₂ and 10% FBS], and cells were washed, fixed, and stained at time points from 15 to 120 min after calcium replacement.

For immunofluorescence, cells were washed briefly three times in cold PBS (2.7 mM KCl, 1.5 mM KH₂PO₄, 137 mM NaCl, 8.1 mM Na₂HPO₄). Cells were fixed for 10 min at room temperature in 3.75% paraformaldehyde, washed three times for 5 min in PBS, and extracted for 5 min in CSK buffer, and conditioned culture medium was used for rr-1 immunoblotting experiments. For calcium replacement experiments, 8-day filter-grown cells were washed once in LCM (DMEM with 5 µM CaCl₂; Refs. 46, 51) and incubated with LCM plus 2.5% dFBS (FBS extensively dialyzed against Tris-saline; see Refs. 46, 51). At time points from 15 to 240 min after calcium removal, cells were washed and fixed immediately for immunofluorescence staining (see below).

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For immunofluorescence, cells were washed briefly three times in cold PBS (2.7 mM KCl, 1.5 mM KH₂PO₄, 137 mM NaCl, 8.1 mM Na₂HPO₄). Cells were fixed for 10 min at room temperature in 3.75% paraformaldehyde, washed three times for 5 min in PBS, and extracted for 5 min in R buffer, containing 1 mM CaCl₂, 1% Nonidet P-40, and 1% Triton X-100 in PBS (61) plus 10 mM PMSF, as equal halves, and frozen at –15 min at 13,000 rpm. Supernatants were collected, aliquoted as equal halves, and frozen at –80°C.

Extracts or immune complexes were separated by SDS-PAGE (33), in 6.25% or 7.5% polyacrylamide gels made from a stock of 30% acrylamide-0.6% bis-acrylamide. For immunoblotting, proteins were transferred to nitrocellulose membrane (Bio-Rad) in a buffer containing 25 mM Tris, pH 8.3, 192 mM glycine, and 20% methanol. Membranes were stained with Ponceau S and blocked overnight at 4°C in TBS-T (10 mM Tris, pH 7.4, 100 mM NaCl, 0.1% Tween 20) plus 5% Carnation nonfat powdered milk and 3% BSA (Sigma). Primary antibodies were diluted (1:5,000–1:10,000) in blocking buffer and incubated with the membrane for 1 h at room temperature with constant rotation. Membranes were washed 5 times for 10 min each wash with TBS-T and then incubated as above with a species-matched horseradish peroxydase-conjugated secondary antibody (Amersham, Arlington Heights, IL) diluted 1:10,000 in blocking solution. Membranes were again washed five times for 10 min each wash with TBS-T, developed by electrochemiluminescence (Amersham), and exposed to film (Kodak Bio-Max ML, Eastman Kodak, Rochester, NY). Bands were scanned with a Silverscanner III (LaCie, Beaverton, OR) and quantified with Biolmage IQ software (Ann Arbor, MI).

Metabolic labeling and immunoprecipitation. For pulse labeling experiments, cells were washed once and then starved for 20 min in methionine- and cysteine-free DMEM (46, 51) supplemented with 2.5% dFBS, then pulse labeled with 0.250 mCi [³⁵S]methionine/cysteine (Pro-mix, Amersham) per filter in the same media for 5, 10, or 15 min. Cells were washed three times in cold PBS and extracted for 10 min on ice with R buffer containing 2.5% dFBS as above for 4, 6, or 8 h.

For pulse-chase labeling experiments, cells were washed, starved, and pulse labeled for 20 min with 0.125 mCi [³⁵S]methionine/cysteine, as above. Filters were then washed twice with DMEM containing 10,000-fold excess of cold methionine and cysteine with 5 mM sodium butyrate and chased in the same media for up to 24 h. At the end of the chase period, cells were washed with PBS and extracted in R buffer as above.

For steady-state labeling, cells were washed once in methionine and cysteine-free media, then incubated for 18–24 h in DMEM supplemented with 2.5% dFBS, 10 μM methionine, 10 μM cysteine, and 0.250 mCi [³⁵S]methionine/cysteine. At the end of the labeling period, cells were washed in PBS and extracted in K buffer as above.

Prepared extracts were preclarified on ice for 15 min with 5 μl of nonimmune serum and 30 μl of Pansorbin cells (Calbiochem, San Diego, CA). Pansorbin cells were removed by centrifugation for 5 min at 13,000 rpm. Primary antibody (4–16 µl) was added to the extracts for at least 1 h on ice for mouse monoclonal antibodies (rr-1, 4A9), a rabbit anti-mouse bridging antibody (DAKO, Carpinteria, CA) was added (10–40 µl). Sixty microliters of protein A Sepharose CL-4B beads (Pharmacia, Uppsala, Sweden) were added and incubated for at least 1 h at 4°C with rotation. Alternatively, rr-1 immune complexes were incubated with 50–100 μl of packed antimouse-coupled Sepharose beads (ICN, Costa Mesa, CA), for
60 min at 4°C with rotation. The protein A or anti-mouse beads were washed three times with extraction buffer (R buffer or K buffer), and 60 µl of SDS sample buffer were added before boiling at 100°C for 10 min. The immunoprecipitates were separated by 6.25 or 7.5% SDS-PAGE as above. Gels were processed for immunoblotting, or, for 35S-labeled samples, gels were processed for fluorography, as described (26). Gels were dried and exposed to Kodak X-OMAT AR film at 280°C. Fluorographs were scanned with a Silverscanner III (LaCie) and bands were quantified with BioImage IQ software. For T151 pulse-chase experiments, gels were quantified using a Molecular Dynamics STORM 820 phosphorimager (Sunnyvale, CA) and ImageQuant software (Molecular Dynamics).

RESULTS

Mutant cadherin fusion protein construction and expression. We constructed chimeric proteins containing transmembrane and cytoplasmic domain sequences of E-cadherin (Fig. 1A). For most experiments, GP2-Cad1 was stably transfected in MDCK cells, and two independent clones were characterized. To engineer the GP2-Cad1 fusion protein, the extracellular domain of
canine E-cadherin was replaced by that of a GPI-linked protein, GP2 (a membrane protein of pancreatic acinar cells; 15, 28). Cells expressing GP2 (13, 35) and parental, untransfected MDCK cells (type II) were used as controls. GP2-Cad1 and GP2 were detected using a monoclonal antibody that recognizes GP2 extracellular domain (4A9; Ref. 13). Immunoblot analysis of stable monoclonal antibody that recognizes GP2 extracellular controls. GP2-Cad1 and GP2 were detected using a tal, untransfected MDCK cells (type II) were used as controls. 15, 28). Cells expressing GP2 (13, 35) and paren-
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A survey of junctional and cytoskeletal proteins (including E-cadherin, α-catenin, β-catenin, ZO-1, occludin, desmoplakin, desmoglein, ankyrin, fodrin, and Na^+-K^+-ATPase) showed that steady-state levels of these proteins were not altered by expressing low levels of GP2-Cad1. However, we found that expression of ZO-1, ankyrin, and Na^+-K^+-ATPase was decreased in the presence of 5 mM sodium butyrate, both in control and GP2-Cad1-expressing cells (data not shown). The expression level of endogenous E-cadherin was also decreased, and was shown to be a specific consequence of increased GP2-Cad1 expression and independent of sodium butyrate (Figs. 2B and 8A; see below). Furthermore, 48 h of 5 mM sodium butyrate treatment resulted in significant morphological changes in established monolayers of both control and GP2-Cad1-expressing cells (Fig. 2B). Cell-cell junction stability and assembly were examined in sodium butyrate-treated cells expressing high levels of GP2-Cad1 and using T151 cells (without sodium butyrate treatment). These data showed that effects were consistent with those seen for low expression (see below). Interpretation of results using GP2-Cad1 cells treated with sodium butyrate was problematic due to the independent sodium butyrate effects, and therefore only data for low-expression cells were shown.

We examined effects of low-level GP2-Cad1 and T151 expression on the stability of cell-cell junctions. Fully polarized MDCK cell monolayers were switched from HCM (1.8 mM Ca^{2+}) to LCM (5 µM Ca^{2+}) to induce junctional complex disassembly. Cells were processed at different times after the switch for double-label immunofluorescence to follow the distribution of adherens junction, tight junction, and desmosome (data not shown) proteins during junctional complex disassembly. To analyze protein distributions, a z-series of x-y sections through the entire volume of the monolayer were collected with a confocal microscope and combined to visualize any alterations in junction organization in all focal planes simultaneously.

Following a 15-min switch to LCM, we observed significant redistribution of E-cadherin and β-catenin (Fig. 3, A and B) in GP2-Cad1 cells, but very minor changes in the distributions of these proteins in control cells. After 30 min in LCM, GP2-Cad1 cells mostly lacked cell contact staining for E-cadherin and β-catenin. However, at this time, the majority of control cells still showed only minor alteration to characteristic adherens junction staining of E-cadherin and β-catenin. By 45 min of LCM treatment, cell-cell contact staining of E-cadherin and β-catenin was completely disrupted in GP2-Cad1 cells, whereas control cultures still had significant cell-cell contact staining for these proteins. It was noted that the distribution of GP2-Cad1 coincided with that of E-cadherin and β-catenin throughout this time course (data not shown).

All experiments described in this paper were repli-
cated using cells expressing a mutant cadherin (T151) under the control of the tet repressor. T151 mutant cadherin protein consists of an HA epitope tag engineered into a nonfunctional, truncated extracellular domain, the transmembrane domain and complete cytoplasmic domain of E-cadherin (Fig. 1A and data not shown). Using these cells, mutant cadherin overexpression was achieved without using sodium butyrate treat-
ment, and levels of mutant cadherin expression could be titrated by incubating cells in various concentra-
tions of doxycycline (which binds the tet repressor protein and blocks its association with the promoter driving mutant cadherin expression). Incubating T151 cells with 0.1 ng/ml doxycycline in culture medium repressed mutant cadherin protein levels to amounts that were similar to GP2-Cad1 protein expression (in cells that were not treated with sodium butyrate).

Effects of mutant cadherin expression on junctional complex stability. To determine whether expression of GP2-Cad1 fusion protein affected cell-cell junction integrity, the distribution of epithelial junctional complex proteins in established cell monolayers of GP2-Cad1 and control cells was analyzed by immunofluorescence. Proteins of the adherens junction (E-cadherin, β-catenin), tight junction (ZO-1, occludin), and desmosome (desmoplakin) were localized at sites of cell-cell contact in GP2-Cad1-expressing cells, in patterns indistinguishable from those in control cells (Fig. 2A). Thus low level expression of GP2-Cad1 had no apparent effect on junctional complex morphology at steady state. Similar results were obtained using T151 cells expressing low levels of mutant cadherin (treated with 0.1 ng/ml doxycycline; data not shown). Significantly,
Cell monolayers expressing GP2-Cad1 showed significant redistribution of tight junction proteins by 15 min after switching cells to LCM (Fig. 4, A and B); large breaks in the continuity of both ZO-1 and occludin cell contact staining were apparent, unlike the staining patterns in control monolayers. By 30 min in LCM, GP2-Cad1 cells showed nearly complete disruption of ZO-1 and occludin staining at cell-cell contacts. In contrast, tight junctions appeared relatively intact in control cells. At 45 min of LCM treatment, GP2-Cad1 cells showed nearly complete redistribution of ZO-1 and occludin from cell-cell contacts. In control cell monolayers, many cells maintained ZO-1 and faint occludin staining in apical belts between cells that were reminiscent of normal tight junction morphology.

Again, equivalent results for adherens junction and tight junction disassembly were obtained using T151 cells expressing low levels of mutant cadherin (0.1 ng/ml doxycycline treated) and completely repressed T151 cells (20 ng/ml doxycycline treated) for controls (data not shown).

Effects of mutant cadherin expression on junctional complex reassembly. The results described above demonstrate that junctional complexes in MDCK cells expressing low levels of GP2-Cad1 or T151 were more rapidly disrupted than in control cells. We investigated whether low levels of mutant cadherin protein expression affected junctional complex reassembly. Confluent MDCK monolayers were incubated in LCM for 2 or 4 h, which is sufficient for complete disassembly of junctional complexes (no junctional complex component staining was observed at sites of cell-cell contact). Results of reassembly experiments using cells incubated in LCM for 2 or 4 h were equivalent. This shows that, operationally, the junctions were completely disassembled after 2 h, because additional low-calcium treatment did not change the outcome of the reassembly experiments. Cultures were switched back to normal medium containing 1.8 mM Ca²⁺ for various times, and processed for double-label immunofluorescence localization of adherens junction, tight junction, and
desmosomal (data not shown) proteins. Optical sections through the whole cell volume were combined to analyze the entire junctional complex.

In control cells, accumulation of E-cadherin at sites of cell-cell contact was evident by 30 min after returning cells to HCM (Fig. 5A). In contrast, cells expressing GP2-Cad1 showed little or no accumulation of E-cadherin staining at cell-cell contact sites at this time. After 60 min in HCM, E-cadherin was present at cell-cell contact sites in nearly all the cells of the control monolayer. However at 60 min, cells expressing GP2-Cad1 exhibited weak, discontinuous E-cadherin staining at cell-cell contacts within a small subset of cells. After 90 min in HCM, the pattern of E-cadherin staining in control cells was approaching that in an established monolayer. In GP2-Cad1 expressing cells, E-cadherin was detected at many cell-cell contact sites, but significantly less than in control cells.

β-Catenin accumulated at sites of cell-cell contact in control cells after 30 min in HCM, although significant cytoplasmic β-catenin staining was also observed (Fig. 5B). By comparison, β-catenin localization in GP2-Cad1 cells was primarily cytoplasmic, with little or no staining at cell-cell contacts. Sixty minutes after the switch to HCM in control cells, β-catenin was enriched at sites of cell-cell contact, like that seen for E-cadherin. In contrast, β-catenin staining was still diffuse in the cytoplasm with little staining at cell-cell contact sites after 60 min of reassembly in GP2-Cad1-expressing cells. After 90 min in HCM, β-catenin staining in control cells was prominent at cell-cell contacts and cytoplasmic staining was largely lost. At the same time, GP2-Cad1

Fig. 3. Reduced adherens junction stability in cell lines expressing the GP2-Cad1 fusion protein. Established monolayers of GP2-Cad1 and control (untransfected) cells were switched to medium containing 5 µM calcium (LCM) for the indicated times, and then processed for immunofluorescence to detect adherens junction proteins. Cells were double-labeled for E-cadherin (A) and β-catenin (B), and each panel is the sum of a series of x-y planes, collected at 0.4-µm intervals, through the entire cell volume. This is a representative experiment of more than 8 independent trials. Bar, 35 µm.
cells showed only weak β-catenin staining at sites of cell-cell contacts and prominent cytoplasmic staining. Tight junctions also assembled more rapidly in control cells than in cells expressing GP2-Cad1 (Fig. 6A and B). After 30 min in HCM, large patches of control cell contact sites showed significant ZO-1 and occludin staining, while there was little or no staining at cell cell contact sites in GP2-Cad1-expressing cells. Control cells showed nearly complete redistribution of ZO-1 and occludin to sites of cell-cell contact after 45 min in HCM. GP2-Cad1-expressing cells showed some ZO-1 and occludin accumulation at cell contact sites. By 60 min in HCM, control monolayers showed a continuous network of ZO-1 and occludin staining at the apical belt at cell contacts throughout the monolayer. By comparison, GP2-Cad1 cells at 60 min in HCM showed discontinuous ZO-1 and occludin staining at cell-cell contacts.

Equivalent results for adherens junction and tight junction reassembly were again obtained using T151 cells expressing low levels of mutant cadherin (0.1 ng/ml doxycycline treated) and completely repressed T151 cells (20 ng/ml doxycycline treated) for controls (data not shown).

Mutant cadherin expression does not alter the composition of the endogenous cadherin-catenin complex. The results described above show that low-level expression of GP2-Cad1 or T151 in MDCK cells significantly accelerated disassembly and retarded reassembly of adherens junctions and tight junctions in cell monolayers. The reduced tight junction stability in cell lines expressing the GP2-Cad1 fusion protein is due to the disruption of cadherin-catenin complexes. The mutant cadherin expression does not alter the composition of the endogenous cadherin-catenin complex.

Fig. 4. Reduced tight junction stability in cell lines expressing the GP2-Cad1 fusion protein.

The images depict the morphology of MDCK cells expressing GP2-Cad1 or control cells treated with doxycycline. The images show the redistribution of β-catenin and ZO-1 at cell-cell contacts in response to calcium withdrawal. The bar in the images represents 35 µm.
junctional complexes. Previous studies using overexpression of analogous mutant cadherin molecules (32, 69) suggested that mutant phenotypes were generated by competition for endogenous catenin binding. Because the level of mutant cadherin expression was ~10% of endogenous cadherin in our experiments, catenin competition mechanisms are not responsible for generating effects on junctional complex stability and reassembly. Total cellular levels of α-catenin and β-catenin were similar in control cells and in cells expressing low levels of GP2-Cad1, and α-catenin, β-catenin, and plakoglobin were detected in GP2-Cad1 immunoprecipitates (data not shown).

To directly examine the possibility that low-level expression of GP2-Cad1 somehow alters the stoichiometry of E-cadherin-catenin complexes, immunoprecipitation was performed on GP2-Cad1 and control cells using the monoclonal antibody rr-1, which specifically recognizes the E-cadherin extracellular domain, and thus only endogenous E-cadherin (22). Immune complexes were separated by SDS-PAGE. Immunoblotting for α-catenin, β-catenin, and plakoglobin revealed that catenin binding to endogenous E-cadherin was similar in GP2-Cad1-expressing cells and control cells (Fig. 7). Similar results were obtained from cells metabolically labeled to steady state with [35S]methionine/cysteine (data not shown).

Overexpression of mutant cadherin reduces the amount and metabolic stability of endogenous E-cadherin. The expression level of GP2-Cad1 fusion protein increased by incubating cells in 5 mM sodium butyrate (Figs. 1D and 8A). Under these conditions, the GP2-Cad1 level was approximately threefold higher than that of endogenous E-cadherin in control cells.

Fig. 5. GP2-Cad1 fusion protein expression retards adherens junction reassembly. Established monolayers of GP2-Cad1 and control (untransfected) cells grown in normal medium were incubated in LCM for 2 h to disassemble junctions. Then, cells were switched back to normal medium (HCM) for the indicated times. Cells were processed for immunofluorescence and double-labeled with antibodies against E-cadherin (A) and β-catenin (B). Each panel is the sum of a series of x-y planes, collected at 0.4-µm intervals, through the entire cell volume. This is a representative experiment of more than 10 independent trials. Bar, 35 µm.
Overexpression of GP2-Cad1 decreased the amount of endogenous E-cadherin at steady state by ~10-fold relative to control cells (Figs. 8A and 2B). However, the levels of α- and β-catenin (Fig. 8B) and stoichiometry of the cadherin-catenin complex were comparable under control and high-level GP2-Cad1 expression conditions (data not shown). This effect on endogenous E-cadherin expression levels was not due to sodium butyrate treatment alone or to overexpression of any exogenous protein from a transfected gene. Both untransfected MDCK cells and MDCK cells transfected with wild-type GP2 had high levels of endogenous E-cadherin in the presence of 5 mM sodium butyrate (Figs. 8A and 2B). Additionally, overexpression of T151 mutant cadherin protein resulted in downregulation of endogenous E-cadherin (Fig. 8C). Repressing mutant cadherin expression levels to low levels (0.1 ng/ml doxycycline treatment) or completely repressing T151 expression (20 ng/ml doxycycline treatment; Fig. 8C) reversed this effect on T151 cells. Thus the decrease in endogenous E-cadherin was a consequence of mutant cadherin expression and not sodium butyrate per se.

To determine whether the decrease in the level of endogenous cadherin was related to the observed effect of mutant cadherin on the plasma membrane junctional complex assembly events, we expressed another mutant cadherin fusion protein that consists of GST fused to the complete E-cadherin cytoplasmic domain (named GST-ECad; Fig. 1A). This fusion protein is a cytoplasmic protein that contains neither a signal sequence nor transmembrane sequence. Overexpressing GST-ECad at very high levels did not affect endogenous E-cadherin levels (Fig. 8C), suggesting that the cellular system for sensing cadherin levels acts at the plasma membrane.
To determine whether the rate of synthesis or the metabolic stability of endogenous E-cadherin was affected by overexpression of mutant cadherin, pulse labeling and pulse-chase labeling experiments were performed to follow synthesis and catabolism of E-cadherin, respectively. Cells were pulse labeled with [35S]methionine/cysteine for 5, 10, and 15 min followed by immunoprecipitation using the monoclonal antibody rr-1, specific for endogenous E-cadherin. Analysis of the immunoprecipitates showed that the rate of E-cadherin synthesis was similar in control and GP2-Cad1 cell lines with or without sodium butyrate induction of GP2-Cad1 expression (Fig. 9A).

To examine the stability of endogenous E-cadherin in GP2-Cad1-expressing cells and control cells, cells induced with sodium butyrate were pulse labeled for 20 min with [35S]methionine/cysteine and chased with medium containing excess methionine and cysteine over a 24-h time course (Fig. 9B). E-cadherin was immunoprecipitated from cell extracts, and the immunoprecipitates were separated by SDS-PAGE. Quantitation of fluorographs showed that the half-life of endogenous E-cadherin was reduced from 6.1 h (± 0.88 h SD, n = 4) in control cells to 3.4 h (± 0.68 h SD, n = 4) in GP2-Cad1-expressing cells. Decreased stability of endogenous E-cadherin is consistent with the reduction in steady-state E-cadherin in cells expressing high levels of mutant cadherin.

These metabolic labeling experiments were also replicated using the T151 cell system. T151 overexpression did not affect the rate of endogenous E-cadherin synthesis (Fig. 9A). However, T151 overexpression reduced the half-life of endogenous E-cadherin from 8.8 h (±0.13 h SD, n = 2) in the presence of 20 ng/ml sodium butyrate to 5.2 h (±0.71 h SD, n = 4) in the absence of sodium butyrate.

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Fig. 7. Cadherin-catenin complex composition was unaltered in the presence of GP2-Cad1 fusion protein. GP2-Cad1 and control (untransfected) cells were extracted and immunoprecipitated with rr-1, a monoclonal antibody that recognizes the extracellular domain of E-cadherin. Immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose, and membranes were probed with antisera to α-catenin (A), β-catenin (B), or plakoglobin (γ-catenin; C). Bars denote 116-kDa and 97-kDa molecular mass standards. These are representative examples of more than 3 independent trials each. Arrowheads denote catenins.

Fig. 8. Expression of high levels of GP2-Cad1 decreased the steady-state level of E-cadherin. A: immunoblot analysis of GP2-Cad1 and control (untransfected) cells with a monoclonal antibody to E-cadherin extracellular domain (top) and polyclonal anti-cadherin cytoplasmic domain antiserum (bottom). GP2-Cad1 expression was induced for 48 h with 5 mM sodium butyrate as labeled (+). Arrow denotes endogenous E-cadherin, and arrowheads indicate the 2 forms of GP2-Cad1 fusion protein (see Fig. 1D). This is a representative example of more than 5 independent experiments. B: immunoblot analysis of GP2-Cad1 cells with anti-β-catenin antisemur (top) and anti-α-catenin antisemur (bottom). GP2-Cad1 expression was induced for 24 h with 5 mM sodium butyrate as labeled (+). This is a representative experiment of more than 5 trials. Controls (untransfected) cells also had equal levels of both α- and β-catenin with or without sodium butyrate treatment (data not shown). C: immunoblot analysis of T151 cells with a polyclonal anti-cadherin cytoplasmic domain antisemur. T151 expression was repressed by incubating cells with different concentrations of doxycycline (Dox) for 3 days. No doxycycline (No Dox) allows overexpression of the mutant cadherin. Low-level expression of T151 (less than endogenous E-cadherin levels) was observed when cells were incubated with 0.1 ng/ml doxycycline, and complete repression was observed when cells were incubated with 20 ng/ml doxycycline. Arrows indicate the position of endogenous E-cadherin (E-cad) and T151 mutant cadherin (T151). Note that endogenous E-cadherin was downregulated only when T151 was overexpressed. D: immunoblot analysis of GST-ECad cells with a polyclonal anti-cadherin cytoplasmic domain antisemur. GST-ECad expression was induced for 24 h with 5 mM sodium butyrate as labeled (+). Arrow denotes endogenous E-cadherin, and arrowhead indicates GST-ECad fusion protein. This is a representative example of 2 independent experiments.
Fig. 9. Overexpression of mutant cadherin does not affect endogenous E-cadherin synthesis, but reduces endogenous E-cadherin metabolic stability. A: GP2-Cad1 or untransfected control cells, with (+) and without (−) sodium butyrate induction, or T151 cells grown in the presence or absence of doxycycline were pulse labeled metabolically with [35S]methionine/cysteine for 5, 10, or 15 min, extracted, and immunoprecipitated with anti-cadherin antibodies (rr-1 for GP2-Cad1 cells and ECAD-B.5 for T151 cells). Immunoprecipitates were separated by SDS-PAGE and processed for fluorography. Arrowhead indicates the 135-kDa cadherin precursor, and arrow indicates mature E-cadherin. Quantitation of fluorographs is presented graphically (right), in arbitrary densitometry units. This is a representative experiment of 3 (GP2-Cad1 cells) or 2 (T151 cells) independent trials.

B: GP2-Cad1 or untransfected control cells treated with 5 mM sodium butyrate for 48 h, or T151 cells grown in the presence or absence of doxycycline were metabolically labeled with a pulse of [35S]methionine/cysteine for 20 min. GP2-Cad1 and untransfected control cells were chased with 5 mM sodium butyrate present. The chase medium contained excess methionine/cysteine. Chase times in hours are indicated. Cells were extracted and immunoprecipitated (using rr-1 ascites for GP2-Cad1 cells and ECAD-B.5 for T151 cells). Immunoprecipitates were separated by SDS-PAGE and processed for fluorography. In GP2-Cad1 cells, the half-life (t½) of endogenous E-cadherin was reduced from 6.1 h (±0.88 h SD, n = 4) in control cells to 3.4 h (±0.68 h SD, n = 4) in GP2-Cad1-expressing cells. In T151 cells, the half-life of endogenous E-cadherin was reduced from 8.8 h (±0.71 h SD, n = 2) in control cells to 2.5 h (±0.12 h SD, n = 2).
doxycycline to 2.5 h (±0.71 h SD, n = 2) without doxycycline present (Fig. 9B), confirming the data collected using GP2-Cad1 cells.

**DISCUSSION**

We have expressed mutant cadherin proteins to examine the consequence on the formation and stability of tight junctions and adherens junctions. Low-level mutant cadherin disrupted dynamic processes of cell-cell junction disassembly and reassembly. Despite a normal level of endogenous E-cadherin, disassembly of adherens junctions and tight junctions in cells expressing mutant cadherin exposed to LCM (5 μm Ca²⁺) was more rapid than in control cells, indicating that established junctional complexes were inherently less stable in the presence of the low level of mutant cadherin. Furthermore, low level of mutant cadherin retarded the reassembly of tight junctions and adherens junctions compared with control cells. These observations extend previous studies demonstrating that junctional complex formation was inhibited under conditions of large-scale blockage of cadherin function (3, 22, 23, 67, 69).

We have conducted detailed analysis of the distribution of the desmosomal marker protein desmoplakin during the process of junctional disassembly and reassembly (data not shown) like that performed for adherens junction and tight junction components. We observed that the time courses of adherens junction and tight junction disassembly and reassembly closely corresponded to one another, but desmosome disassembly and reassembly lagged behind adherens junction and tight junction disassembly and reassembly in control, GP2-Cad1, and T151 cells. This suggests that adherens junction and tight junction assembly processes are somehow coupled.

Despite the effects on junctional complex stability and assembly, low-level expression of mutant cadherin allows establishment of polarized MDCK cell monolayers. With time, cell-cell junctions formed that were morphologically indistinguishable from those in control cells, when distributions of tight junction (ZO-1 and occludin), adherens junction (E-cadherin and β-catenin), and desmosome (desmoplakin) marker proteins were examined. Cells expressing a low level of mutant cadherin also developed a measurable transepithelial resistance and restricted inulin passage similar to control cell monolayers.

How does mutant cadherin expression alter junctional complex integrity? Previous notions were that overexpressed mutant (“dominant negative”) cadherin proteins sequester catenins from endogenous cadherins (32, 69). However, this mechanism is unlikely in cells expressing low levels of mutant cadherin. Mutant cadherins expressed at approximately one-tenth the level of endogenous cadherin are too low to support a catenin competition mechanism. In immunoprecipitation experiments, low-level mutant cadherin expression did not alter the extent or stoichiometry of catenin binding to endogenous E-cadherin. The possibility remains that the observed effects on junctional complexes are due to competition for some other unidentified component of the cadherin complex, present in limiting quantities, as suggested by Fujimori and Takeichi (14). Because catenin binding to endogenous E-cadherin was shown to be unaltered in the presence of low levels of mutant cadherin, we suggest that GP2-Cad1 and T151 proteins interfered structurally with endogenous E-cadherin packing in forming cell-cell adhesion junctions. GP2-Cad1 and endogenous E-cadherin colocalized at sites of cell-cell contact (Fig. 1E and data not shown). Studies suggest that cadherin extracellular domain dimers form lateral associations and intercalate with dimers on adjacent cells to build a lattice of cadherin-adhesive interactions (8, 43, 48, 57, 68). We suggest that the juxtaposition of mutant cadherin molecules and endogenous E-cadherin at adherens junctions, even at low expression levels, disrupts cadherin spacing and substitutes nonfunctional binding partners within the extracellular cadherin lattice, producing the observed structural weakness of the junctions. Our data indicate that GP2-Cad1 interferes with cell junction dynamics via a structural effect on cadherin packing, instead of altering cadherin-catenin complex composition as suggested in previous studies (32, 69).

At high levels of GP2-Cad1 or T151 expression (approximately threefold greater than endogenous E-cadherin), steady-state endogenous E-cadherin levels were downregulated. This could be attributed in part to decreased metabolic stability of endogenous E-cadherin. Previous studies have shown little change in E-cadherin half-life even under low calcium growth conditions (E. Shore, L. Hinck, and W. J. Nelson, unpublished results). However, it has been demonstrated that cytoskeletal association may prevent turnover of other membrane-cytoskeletal proteins (24, 45). Mutant cadherin could occupy cytoskeletal-binding sites normally available to endogenous E-cadherin, forcing more rapid turnover of endogenous E-cadherin molecules. Zhu and Watt (69) observed that a mutant cadherin construct lacking the catenin binding domain no longer caused downregulation of endogenous cadherin in keratinocytes. Taken together, these data indicate that the reduced half-life of endogenous E-cadherin, contributing to decreased steady-state E-cadherin levels, is dependent on catenin binding. However, overexpressing a cytoplasmic fusion protein that contains the entire cadherin cytoplasmic domain but no transmembrane sequence (GST-E-Cad) had no effect on endogenous E-cadherin levels (Fig. 8C), suggesting that catenin binding and plasma membrane localization are necessary for regulating cadherin levels. In squamous carcinoma cells, levels of E- and N-cadherin were reciprocally regulated by an unknown mechanism (29), and our results may help explain the observed phenomenon.

The observed downregulation of endogenous E-cadherin in conditions of mutant cadherin overexpression is in contrast to observations of Fujimori and Takeichi (14) and Amagai et al. (3), who found no decrease in endogenous cadherin expression in the
presence of high levels of a mutant cadherin protein. It is interesting to note that in these other two studies the cytoplasmic domain of the mutant protein did not match that of the endogenous cadherin family member (mutant N-cadherin was overexpressed, whereas keratinocytes express E- and P-cadherin). Like our results, Zhu and Watt (69) found that endogenous cadherin was downregulated in the presence of a mutant cadherin molecule with an identical cytoplasmic domain. These data point to the existence of a posttranslational, cellular mechanism for regulating cadherin protein levels.

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REFERENCES