Differential Targeting of T- and N-cadherin in Polared Epithelial Cells*

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To test whether glycosyl phosphatidylinositol-linked T-cadherin is a component of cell junctions like classical cadherins, we have examined its distribution and targeting in polarized epithelial cells. In vivo, T-cadherin was detected on the apical cell surface of the chick intestinal epithelium. In cultures of transfected Madin-Darby canine kidney cells, T-cadherin was also expressed apically, whereas classical N-cadherin resided basolaterally. Both cadherins were directly targeted to their respective membrane domains. Mutant proteins were expressed in Madin-Darby canine kidney cells to identify the regions responsible for differential cadherin localization. NAcyt, an N-cadherin cytoplasmic domain deletion mutant, was stably distributed basolaterally. This mutant was transported to both the apical and basolateral membrane compartments, followed by preferential removal from the apical surface. T-NAcyt, a T-cadherin mutant with the N-cadherin cytoplasmic domain deletion, was localized basolaterally, whereas N-TGPI, a GPI-anchored N-cadherin mutant, resided at the apical domain. The T-cadherin carboxyl-terminal 76 amino acids contain the apical targeting signal and include the signal for GPI anchor attachment. Basolateral localization of N-cadherin is achieved through targeting signals in the cytoplasmic domain. Thus, GPI-linked T-cadherin is not a component of cell junctions, consistent with a function as a recognition rather than a cell adhesion molecule.

Polarized epithelia separate biological compartments and regulate the vectorial transport of ions and solutes. Epithelial cells are polarized into an apical and basolateral pole (1), each characterized by a distinct protein and lipid composition. The induction of epithelial cell differentiation and polarization and the molecular signals responsible for selective protein sorting to distinct membrane domains can be studied in vitro using MDCK1 cells (2).

The organization of MDCK cells into an apical and a basolateral pole depends on extracellular matrix and cell-cell interactions (3–5). Formation of cell-cell contacts, induced through adhesive interactions mediated by E-cadherin (6), results in the differentiation of MDCK cells into a polarized epithelium and the gradual restriction of specific proteins to the apical or basolateral membrane domain (5–8). E-cadherin is a classical transmembrane, calcium-dependent cadherin cell adhesion molecule, which is characterized by five extracellular structural repeats and a highly conserved cytoplasmic region. The cadherin cytoplasmic region interacts with the catenins, a group of cytoplasmic proteins that connects cadherins with the actin-based cytoskeleton (9, 10). In epithelial cells in vitro, E-cadherin is a major constituent of adherens junctions, where it mediates calcium-dependent adhesion and links cortical actin filaments between adjacent cells (7, 11).

T-cadherin is a member of the cadherin family that shares the ectodomain organization with classical cadherins and is anchored to the membrane through a GPI moiety (12, 13). In contrast to classical cadherins that generally do not mediate adhesive interactions without their conserved cytoplasmic domain (14, 15), T-cadherin induces calcium-dependent, homophilic aggregation between transfected cells (13). In the animal, T-cadherin is a component of specific cell populations both within and outside the nervous system. In the nervous system, T-cadherin demarcates specific neuron populations and axon pathways (12, 16, 17), suggesting a role in axon patterning. Outside the nervous system, T-cadherin is expressed on skeletal muscle surfaces and is specifically excluded from myoneural junctions (17), which are demarcated by N-cadherin (19). The mechanisms of how T-cadherin functions in mediating cell-cell interactions and axon guidance are not understood.

To gain insights into the principal function of T-cadherin, we have examined whether T-cadherin is localized to cell-cell junctions like classical cadherins. Two approaches were used: immunohistochemical staining of the chick intestinal epithelium in vivo and heterologous expression of T-cadherin in polarized MDCK cells in vitro. Our results revealed T-cadherin on apical cell surfaces of epithelial cells, in contrast to the basolateral localization of classical cadherins. Examination of the mechanisms responsible for differential cadherin localization identified direct targeting as well as selective removal from specific membrane domains as key principles.

Experimental Procedures

Cells and Tissue Culture—Madin-Darby canine kidney cells, clone II/8, were obtained from Dr. James Nelson (Stanford University, Stanford, CA) (20) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin.

Cryosectioning of Chick Embryonic Tissue—Fertilized White Leghorn chicken eggs (McIntyre Poultry Farm, Lakeside, CA) were incubated in a force-draft incubator until the desired developmental stages. Embryos were dissected in phosphate-buffered saline (PBS, pH 7.2), fixed for 6 h in 4% formaldehyde in PBS, and rinsed in several changes of PBS. The tissue was cryoprotected overnight in 30% sucrose in PBS.
Dours-Zimmermann (12)), was generated by reverse transcription-

T-cadherin (amino acids 615–690, numbering according to Ranscht and below.

Coated slides, and labeled with anti-T-cadherin antiserum as described below.

The cells were incubated with 50 mM NH₄Cl in PBS/CM for 30 min at room temperature, followed by rabbit peroxidase-antiperoxidase for 1 h at room temperature (1:100; Sternberger Monoclonals, Baltimore, MD). All of the immunoreagents were diluted in TBS containing 1% goat serum and 0.1% Photo-flo. The cultures were then labeled with goat anti-rabbit IgG (1:50; Cappel) for 1 h at room temperature, followed by rabbit peroxidase-antiperoxidase for 1 h at room temperature.

The cells were then fixed in 2% OsO₄ and 15% potassium ferricyanide in 0.1 M sodium cacodylate, pH 7.4, and dehydrated through a series of ethanol, flat embedded in a TAAB/Epon (1:1) resin embedding mixture, and polymerized for 2 days at 65°C. Ultrathin sections were cut and examined with a Hitachi 600E transmission electron microscope.

Immunoblotting—Cells from a confluent 35-mm plate were lysed for 20 min at 4°C in 100 μl of lysis buffer (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 50 mM leupeptin, 1 mM aprotinin, and 1 mM phenylmethylsulfonyl fluoride) and then boiled for 1 h. Insoluble material was pelleted, and 14 μl of the supernatant was analyzed by Western blotting of proteins separated by SDS-PAGE and transferred onto Immobilon polyvinylidene difluoride membranes (Millipore Intertech, Bedford, MA; Ref. 23). The blots were blocked for 1 h at room temperature with 5% milk in Tris-buffered saline (TBS; 0.1 M Tris and 77 mM NaCl) and then incubated with rabbit anti-T-cadherin (1:1,000, mouse anti-N-cadherin (1:500), or rat anti-N-cadherin (NCD2, 1:200; a gift from M. Takeichi, Kyoto University, Kyoto, Japan; Ref. 21) in TBS for 1 h at room temperature. Primary antibodies were detected by chemiluminescence (ECL system, Amersham Life Science, Inc.) after incubation of the blots with horseradish peroxidase-conjugated sheep anti-rabbit, sheep anti-mouse, or sheep anti-rat IgG, respectively.

Phosphatidylinositol-specific Phospholipase C Release—T-cadherin was removed with phosphatidylinositol-specific phospholipase C (a gift from M. G. Low, Columbia University, New York, NY) from MDCK and Cos7 cells as described previously (13). Proteins were separated by SDS-PAGE and analyzed by immunoblotting with anti-T-cadherin or anti-N-cadherin antibodies as described above.

Metabolic Labeling—For metabolic labeling, MDCK cell cultures grown on Transwell™ filters were rinsed twice with PBS. The cells were incubated with DMEM/FBS without methionine for 15 min at 37°C. [35S]Methionine/cysteine (250 μCi; DuPont NEN) was added to the basolateral side of cells on the Transwell™ filter in a total volume of 0.2 ml of DMEM/FBS without methionine (1 ml) was added to the apical compartment to keep the cells submerged. Metabolically labeled proteins were biotinylated for targeting assays as described below. For assays measuring cadherin removal from specific membrane domains, cells were incubated in DMEM/FBS without methionine for 15 min, labeled with [35S]Methionine/cysteine as above, and chased for the indicated times in cold DMEM/FBS.

Biotinylation and Immunoprecipitation—Biotinylation was performed essentially as described by Rodriguez-Boulan et al. (24). Briefly, MDCK cells were plated at high density on 24-mm Costar Transwell™ plates (as described above). After 6–8 days in culture, the permeability of the monolayer was measured by adding 0.2 μl of [3H]inulin to the apical compartment of the filter chamber. Chambers were washed with PBS/C at 2 h after 37°C. [3H]Inulin was measured in the basolateral compartment. If more than 1% of the added inulin permeated, the monolayers were discarded. Monolayers with less than 1% inulin permeability were washed with PBS/C and biotinylated by adding 0.5 mg sulfo-NHS-biotin (stock solution, 200 mg/ml in dimethylsulfoxide; Pierce) in 1 ml of PBS/C either to the apical or basolateral compartment of the filter chamber. Components not receiving sulfo-NHS-biotin were filled with an equal volume of PBS/C. After 30 min of agitation at 4°C, filters were washed three times with Tris-saline/phenylmethylsulfonyl fluoride (15 mM Tris, pH 7.5, 120 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride) and extracted with 100 μl of lysis buffer (1% Nonidet P-40, 60 mM octylglucoside, 10 mM Tris, pH 8.0, 150 mM NaCl, 2 mM CaCl₂, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 50 μM leupeptin, and 4 μg/ml aprotinin). The cells were scraped from the filter with a policeman and sedimented in a microfuge for 5 min.

Immunoprecipitation was essentially performed as described by Wolner et al. (8). Samples were analyzed by SDS-PAGE, and gels were processed for fluorography using Amplify (Amersham). Protein bands were quantitated by scanning densitometry (Pharmacia).
resistant colonies, enriched by fluorescence-activated cell sort-

lines expressing either cadherin were selected from neomycin-

causeMDCKcellsexpressendogenousE-cadherin(28,29).Cell

expression plasmids and pSV2 neo. N-cadherin was chosen be-

with either chicken T-cadherin (13) or N-cadherin cDNA ex-

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Differential Targeting of T- and N-cadherin in Polarized Epithelial Cells in Vitro—To address whether heterologously expressed, GPI-anchored T-cadherin and classical cadherins are differentially targeted in polarized epithelial cells in vitro, MDCK cells were stably transfected with either chicken T-cadherin (13) or N-cadherin cDNA expression plasmids and pSV2 neo. N-cadherin was chosen because MDCK cells express endogenous E-cadherin (28, 29). Cell lines expressing either cadherin were selected from neomycin-resistant colonies, enriched by fluorescence-activated cell sorting, and grown to confluence on collagen-coated Transwell® polycarbonate filters. Cadherin distribution was examined by confocal microscopy following indirect immunostaining with rabbit anti-chick T-cadherin antiserum (12) and monoclonal anti-N-cadherin antibody GC4. In optical sections in the vertical plane (XZ section), T-cadherin was only apparent on apical MDCK cell surfaces (Fig. 2A). In contrast, endogenously expressed E-cadherin, detected with monoclonal anti-canine E-cadherin antibody rr1 (22), was expressed at the lateral surface both in wild type MDCK cells as described previously (28, 29; not shown) and in T-cadherin-transfected cells (Fig. 2B). Exclusive T-cadherin localization on the apical cell surface was confirmed by immuno-electron microscopy after immuno-peroxidase staining (Fig. 2C).

To verify that the anticipated GPI-anchored T-cadherin protein is expressed in MDCK cells, transfected cells were treated with phosphatidylinositol-specific phospholipase C (PI-PLC), an enzyme that specifically cleaves GPI-linked anchors (30). PI-PLC released a large fraction of T-cadherin from the cell surface into the culture medium (Fig. 2D). Some proportion of T-cadherin was resistant to PI-PLC treatment, possibly due to incomplete digestion. In controls without enzyme, T-cadherin remained associated with cell membranes.

T- and N-cadherin are expressed in an overlapping pattern on cultured sympathetic neurons and immature muscle cells (17, 31), raising the possibility that one cadherin affects the localization of the other at specific membrane domains. To test this possibility, MDCK cells were double transfected to stably express T- and N-cadherin protein. Both 95-kDa mature T-cadherin and 120-kDa N-cadherin were detected by Western blotting (not shown). As in the singly transfected cells, T-cadherin was localized to the apical surface (Fig. 3A), whereas N-cadherin was detected basolaterally (Fig. 3B). Apical T-cadherin and basolateral N-cadherin expression was already prominent on subconfluent groups of nonpolarized MDCK cells, which are much flatter in morphology (not shown). As the distribution of T- and N-cadherin in the double transfected cells was indistinguishable from that in the single transfected cells, these experiments provide evidence that the localization of these cadherins is independent of each other.

The localization of T-cadherin was biochemically quantitated by immunoprecipitation of surface biotinylated proteins from either the apical or basolateral membrane domain. MDCK cell monolayers with <1% permeability for the [3H]inulin tracer were used for these experiments. Confluent monolayers of T-cadherin-transfected cells were labeled with sulfo-NHS-biotin (a tight junction-impermeable probe) from either the apical or the basolateral side of the filter compartment and extracted with lysis buffer containing 1% Nonidet P-40. As GPI-linked proteins are insoluble in nonionic detergents such as Nonidet

FIG. 1. Apical distribution of T-cadherin in chick intestine. A, frozen sections of embryonic day 21 chick embryo intestine were immuno-

histochemically stained for T-cadherin. T-cadherin is expressed on the apical surface of columnar epithelial cells. B, nuclei of the same villi were stained with propidium iodide to reveal single cells. Arrows, apical surface of epithelial cells.

FIG. 2. Steady state distribution of transfected T-cadherin at the apical surface of MDCK cells grown on polycarbonate filters. Indirect immunofluorescence and confocal microscopy of T-cadherin (A) and endogenous E-cadherin (B) in T-cadherin-transfected MDCK cells. A and B, top panels, Z sections of the monolayer (line). T-cadherin is localized on the apical membrane surface, whereas E-cadherin is localized to sites of cell-cell contact. C, electron micrograph of immunostained T-cadherin in MDCK cells grown on a polycarbonate filter. T-cadherin is localized on apical membrane domains Arrows, apical side; arrowheads, lateral side; N nucleus; F, membrane filter. D, release of T-cadherin from the surface of transfected MDCK cells with PI-PLC. T-cadherin-transfected cells were treated with PI-PLC, and cells lysates (cells) and culture medium (SN) were analyzed by Western blotting. With PI-PLC treatment, T-cadherin is released into the culture medium. In controls (no enzyme) T-cadherin remains associated with cell membranes. Mock-transfected cells do not express T-cadherin (lane 1).
P-40 (32–35), T-cadherin was immunoprecipitated from both Nonidet P-40-soluble and Nonidet P-40-insoluble fractions. Precipitated proteins were separated by SDS-polyacrylamide gel electrophoresis, and biotinylated T-cadherin was detected with peroxidase-coupled streptavidin on immunoblots (Fig. 4). T-cadherin was predominantly (93%) localized on the apical cell surface (Fig. 4), and approximately 50% of the protein was soluble in Nonidet P-40 extracts (Fig. 4, lane 1), whereas the remainder was resistant to nonionic detergent extraction (Fig. 4, lane 3). A small percentage of T-cadherin (7%) was localized basolaterally, probably due to misrouting. Thus, both immunohistochemical and biochemical analyses localize the majority of T-cadherin at the apical MDCK cell surface.

Wild Type N-Cadherin and Cytosplasmic Tail Deletion Mutant NΔcyt Are Localized at the Basolateral Domain in MDCK Cells—Classical cadherins contain in their cytoplasmic domain signals for basolateral sorting (50). To investigate whether deletion of these signals is sufficient to shuttle cadherins apically like cytoplasmic tail deletion mutants of the low density lipoprotein receptor (37), we have generated and tested for localization and targeting an N-cadherin cytoplasmic tail deletion mutant, NΔcyt (Fig. 5). The NΔcyt protein lacks all but three amino acids (KRR) of the N-cadherin cytoplasmic domain. The localization of the NΔcyt mutant was analyzed by confocal microscopy of confluent, stably transfected MDCK cell monolayers stained by indirect immunofluorescence. Optical sections in the Z axis revealed the majority of the NΔcyt protein on basolateral MDCK cell surfaces (Fig. 6A). To biochemically quantitate NΔcyt protein distribution, monolayers of MDCK cells were biotinylated from either the apical or basolateral surface, extracted with lysis buffer containing 1% Nonidet P-40, and immunoprecipitated with monoclonal anti-N-cadherin antibodies. The majority (87%) of the biotinylated NΔcyt protein was detected in extracts biotinylated from the basolateral surface (Fig. 6B, NΔcyt). This distribution was closely similar to that of wild type N-cadherin (Fig. 6B, N). Thus, in contrast to the prominent apical localization of deletion mutants of other basolaterally targeted proteins, NΔcyt was stably expressed on the basolateral domain.

**T- and N-cadherin Are Directly Targeted to Distinct Membrane Domains in MDCK Cells**—To determine whether newly synthesized T- and N-cadherin are directly sorted to the apical or basolateral membrane, the kinetics of cadherin arrival at each membrane domain were measured between 4 and 96 h after induction of cell-cell contact. For synchronization, MDCK cells were kept on TranswellTM polycarbonate filters in Dulbecco’s modified Eagle’s medium (DMEM) F12/15% fetal bovine serum under low Ca2+ conditions (37). Formation of intercellular contacts was induced by raising the Ca2+ concentration to 1.8 mM. At various times after Ca2+ induction, cells were labeled for 1 h with [35S]methionine/cysteine and biotinylated from either the apical or basolateral filter compartment (see above), and cadherins were immunoprecipitated with specific antibodies. The immune complexes were dissociated, and biotinylated cadherins were identified in either the apical or the basolateral fraction by reprecipitation with streptavidin-agarose. After 4 h, 84% of total T-cadherin was detected on the apical membrane domain (Fig. 7A). With further polarization, the percentage of T-cadherin delivered to the apical membrane increased, reaching a maximum of 96% after 96 h (Fig. 7A). Thus, T-cadherin was directly targeted to the apical membrane domain already 4 h after induction of cell-cell contact.

Targeting assays of wild type N-cadherin in transfected MDCK cells demonstrated that 86% (96 h after induction of cell-cell contact) of newly synthesized N-cadherin was delivered directly to the basolateral surface of fully polarized cells (Fig. 7B, N). This is comparable with the targeting of endogenous E-cadherin in MDCK cells, 82% of which is directly transported to the basolateral membrane domain 96 h after induction of cell-cell contact (Fig. 7B, E). A small percentage of N- and E-cadherin is missorted to the apical surface. As apically transported wild type N- and E-cadherin do not accumulate at the apical surface, they must be rapidly removed, either by proteolytic degradation or transcytosis.

**N-Cadherin Cytosplasmic Tail Deletion Mutant NΔcyt Is Transported to the Apical and Basolateral Domain in MDCK Cells and Is Removed at the Apical Surface**—To address whether the NΔcyt mutant protein is directly targeted to the basolateral domain, the arrival of metabolically labeled NΔcyc...
protein at each membrane surface was measured in targeting assays as described above. Analysis of proteins at both membrane domains revealed that mutant protein was transported in similar amounts to both the apical and basolateral membrane domains (Fig. 7B, NΔcyt).

The random transport of the NΔcyt mutant raised the possibility that this protein is preferentially removed from the apical membrane surface. To test this hypothesis, the residence time of newly synthesized protein at each membrane domain was determined. Monolayer cultures were metabolically labeled for 15 min or 1 h with [35S]methionine/cysteine, and the label was chased with medium containing an excess of unlabeled methionine. At various time points during the chase period, duplicate cultures were biotinylated for 15 min at 4°C from either the apical or basolateral filter chamber as described above. Cells were extracted with Nonidet P-40 lysis buffer and immunoprecipitated with monoclonal anti-N-cadherin antibody by Western blotting of transfected cell lysates from either the apical or basolateral filter chamber as described above. Cells were extracted with Nonidet P-40 lysis buffer and immunoprecipitated with monoclonal anti-N-cadherin antibody by Western blotting of transfected cell lysates.

T-Cadherin Carboxy-terminal Amino Acids Are Responsible for Apical Targeting—The regions within N- and T-cadherin responsible for their respective basolateral and apical distribution in MDCK cells were examined by studying the distribution of additional mutants (see Fig. 5).

Expression of T-NΔcyt, composed of the T-cadherin ectodomain and the N-cadherin transmembrane domain and truncated cytoplasmic domain, was achieved only in a small number of cells and was unstable. Examination by indirect immunofluorescence revealed that the T-NΔcyt mutant was localized on the basolateral MDCK cell surface (Fig. 8), identical to that of the NΔcyt protein. Expression of the reverse chimera, N-TGPI, containing the N-cadherin extracellular portion followed by the 76 T-cadherin carboxy-terminal amino acids, was restricted to the apical membrane domain, as shown by confocal microscopic analysis of transfected cells after indirect immunofluorescence (Fig. 9A). As expression of the N-TGPI mutant was unstable in MDCK cells, membrane attachment of the mutant protein was examined after transient transfection of the corresponding DNA construct into Cos7 cells. Proteins of 95 and 110 kDa were detected with monoclonal anti-N-cadherin antibody by Western blotting of transfected cell lysates (Fig. 9B). The sizes of these proteins correspond to those of T-cadherin, which comprises the mature 95-kDa protein and the unprocessed 110-kDa precursor (12, 13). Treatment of N-TGPI expressing Cos7 cells with PI-PLC released both the 95-
and 110-kDa proteins from the cell surface into the culture medium, whereas in controls without PI-PLC, chimeric proteins were associated with cell membranes (Fig. 9B). Thus, the 76 carboxyl-terminal amino acids of T-cadherin include the signal for GPI anchor attachment.

Taken together, these results demonstrate that the carboxyl-terminal 76 T-cadherin amino acids contain the signal for GPI anchor attachment and are responsible for apical targeting of T-cadherin in MDCK cells.

DISCUSSION

In this article, we have examined whether GPI-linked T-cadherin is a component of cell-cell junctions like classical cadherins. In contrast to the basolateral distribution of classical cadherins, T-cadherin was transported and stably expressed at the apical domain of polarized epithelial cells. Apical targeting signals reside within the carboxyl-terminal 76 amino acids of T-cadherin and contain signals for GPI anchor attachment. The signal for GPI-linkage generally consists of an uncharged amino acid followed by a stretch of hydrophobic amino acid residues (38, 39). The T-cadherin carboxyl-terminal 76 amino acid region when fused to the ectodomain of N-cadherin produces a GPI-linked protein that is distributed apically. Therefore, the T-cadherin carboxyl-terminal amino acids are sufficient for GPI anchor attachment and apical targeting.

Classical cadherins contain in their cytoplasmic domain a putative basolateral targeting signal (50) that resembles that of the low density lipoprotein receptor and other basolaterally targeted proteins (36). The targeting motif comprises a tyrosine and a downstream cluster of three negatively charged amino acid residues (38, 39). The T-cadherin carboxyl-terminal 76 amino acid region when fused to the ectodomain of N-cadherin produces a GPI-linked protein that is distributed apically. Therefore, the T-cadherin carboxyl-terminal amino acids are sufficient for GPI anchor attachment and apical targeting.

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to serve as a calcium store and contribute to specific cardiac functions.

The apical localization of T-cadherin in MDCK cells correlates with its apical expression on intestinal epithelial cells in vivo. The highest T-cadherin concentration was detected on the apical extrusion zone where enterocytes exfoliate. Expression on the apical domain is complementary to that of E-cadherin (25, 26) and LI-cadherin (27), which occupy the basolateral contact zones, excluding junctional regions, respectively.

What is the possible function of T-cadherin at the apical surface of epithelial cells? One hypothesis is that T-cadherin is clustered in caveolae as in the heart and serves as a calcium store. Alternatively, and perhaps in addition, T-cadherin may play a role in receiving and transducing signals from the luminal space. A number of GPI-linked proteins of lymphocytes store. Alternatively, and perhaps in addition, T-cadherin may function as a calcium store and contribute to specific cardiac functions.

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2 B. Ranscht, unpublished observation.