

LI-Cadherin-mediated Cell-Cell Adhesion Does Not Require Cytoplasmic Interactions

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Abstract. The adhesive function of classical cadherins depends on the association with cytoplasmic proteins, termed catenins, which serve as a link between cadherins and the actin cytoskeleton. LI-cadherin, a structurally different member of the cadherin family, mediates Ca^{2+} -dependent cell-cell adhesion, although its markedly short cytoplasmic domain exhibits no homology to this highly conserved region of classical cadherins. We now examined whether the adhesive function of LI-cadherin depends on the interaction with catenins, the actin cytoskeleton or other cytoplasmic components. In contrast to classical cadherins, LI-cadherin, when expressed in mouse L cells, was neither associated with catenins nor did it induce an upregulation of β -catenin. Consistent with these findings, LI-cadherin was not resistant to detergent extraction and did not induce a reorganization of the actin cytoskeleton. However, LI-cadherin was still able to mediate Ca^{2+} -

dependent cell-cell adhesion.

To analyze whether this function requires any interaction with proteins other than catenins, a glycosyl phosphatidylinositol-anchored form of LI-cadherin (LI-cadherin^{GPI}) was constructed and expressed in *Drosophila* S2 cells. The mutant protein was able to induce Ca^{2+} -dependent, homophilic cell-cell adhesion, and its adhesive properties were indistinguishable from those of wild type LI-cadherin. These findings indicate that the adhesive function of LI-cadherin is independent of any interaction with cytoplasmic components, and consequently should not be sensitive to regulatory mechanisms affecting the binding of classical cadherins to catenins and to the cytoskeleton. Thus, we postulate that the adhesive function of LI-cadherin is complementary to that of coexpressed classical cadherins ensuring cell-cell contacts even under conditions that downregulate the function of classical cadherins.

CADHERINS are a multifunctional family of transmembrane glycoproteins mediating Ca^{2+} -dependent adhesion of adjacent cells in a homophilic manner (Takeichi, 1988, 1991; Geiger and Ayalon, 1992; Kemler, 1993). Members of this family have been reported to be involved in morphogenesis (Takeichi, 1995), the development of junctional complexes and cell polarity (Nelson, 1992), invasiveness and metastasis (Birchmeier and Behrens, 1994), and most recently, transmembrane transport (Dantzig et al., 1994; Thomson et al., 1995).

Classical cadherins are composed of a highly conserved cytoplasmic domain of ~ 160 amino acids, a single transmembrane domain, and a large extracellular portion that is organized in a series of five structurally related tandem

repeats (Ranscht, 1994). The conserved intracellular domain of classical cadherins is known to associate with a group of cytoplasmic proteins, termed catenins (Ozawa et al., 1989), which serve as a link between cadherins and the cortical cytoskeleton (Hirano et al., 1987). As demonstrated by several experiments, the formation of complexes with catenins is essential for cadherins to function as adhesion molecules. First evidence for the crucial role of this association came from studies, in which cadherins were rendered nonfunctional by COOH-terminal truncations affecting the catenin-binding site (Nagafuchi and Takeichi, 1988, 1989; Ozawa et al., 1989, 1990). Furthermore, in nonadhesive PC9 cells lacking α -catenin, strong cell-cell adhesion could be restored by transfection with α -catenin cDNA indicating that the expression of α -catenin is required for the adhesive function of cadherins (Hirano et al., 1992). α -Catenin is homologous to vinculin (Herrenknecht et al., 1991; Nagafuchi et al., 1991) and is a candidate for linking the cadherin/catenin complex to the actin-based cytoskeleton (Ozawa et al., 1990; Nagafuchi et

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al., 1994). β -Catenin exhibits homology to plakoglobin, a component of desmosomal plaques and adherens junctions (Cowin et al., 1986), and to the product of the *Drosophila* segment polarity gene *armadillo* (McCrea et al., 1991; Butz et al., 1992; Peifer et al., 1992). The primary structure of γ -catenin has not yet been established, but there is growing evidence that it might be identical to plakoglobin (Knudsen and Wheelock, 1992; Peifer et al., 1992; Piepenhagen and Nelson, 1993). Like the armadillo protein, β -catenin is thought to be involved in signal transduction and developmental patterning (reviewed by Gumbiner, 1995; Kühl and Wedlich, 1996). Recent studies suggested that β -catenin might be a target molecule for the regulation of cadherin function, since epithelial cells transformed with the v-Src tyrosine kinase acquired a more mesenchymal morphology, that was correlated with a strong phosphorylation of β -catenin and the perturbation of cadherin activity (Matsuyoshi et al., 1992; Behrens et al., 1993; Hamaguchi et al., 1993). A similar change in morphology could be induced by treatment with EGF or hepatocyte growth factor/scatter factor, which caused tyrosine phosphorylation of β -catenin as well as of plakoglobin (Weidner et al., 1990; Shibamoto et al., 1994). These observations suggest that tyrosine phosphorylation of catenins affects cadherin-mediated cell-cell adhesion.

Recently, LI-cadherin was characterized as a novel member of the cadherin family specifically expressed in polarized epithelia of liver and intestine (Berndorff et al., 1994). In intestinal epithelial cells, LI-cadherin is evenly distributed over the lateral contact zones but is excluded from adherens junctions, whereas coexpressed E-cadherin is concentrated in this specialized membrane region. LI-cadherin exhibits an unusual structure, since its extracellular domain is composed of seven cadherin-type repeats instead of five typical for classical cadherins. In addition, its short cytoplasmic domain consists of only 20 amino acids exhibiting no homology to this highly conserved region of classical cadherins. Nevertheless, LI-cadherin was shown to act as a functional Ca^{2+} -dependent cell adhesion molecule when expressed in *Drosophila* S2 cells (Berndorff et al., 1994).

The strikingly divergent structure of the cytoplasmic domain of LI-cadherin prompted us to investigate whether this region is of similar importance for the adhesive function of LI-cadherin as it is for classical cadherins. The general relevance of this question is emphasized by the recent discovery of two cadherins, HPT-1 (Dantzig et al., 1994) and Ksp-cadherin (Thomson et al., 1995), which are homologous to LI-cadherin, and may thus together constitute a new subfamily of cadherins. Our results show that LI-cadherin is neither associated with catenins, nor is it tightly connected to the actin-based cytoskeleton. Nevertheless, LI-cadherin is able to mediate Ca^{2+} -dependent cell-cell adhesion of transfected L cells even after disruption of the actin cytoskeleton. We were able to demonstrate that the adhesive properties of LI-cadherin are fully retained in a construct, in which the transmembrane and

the cytoplasmic domain have been exchanged for a glycosyl phosphatidylinositol (GPI)¹ anchor. Apparently, the cell-cell adhesion mediated by LI-cadherin is independent of any direct interactions with cytoplasmic components. Since it cannot be affected by the same mechanisms and interactions controlling the function of classical cadherins, we assume that the adhesive function of LI-cadherin is complementary to that of coexpressed classical cadherins.

Materials and Methods

Materials and Antibodies

Rabbit polyclonal anti-LI-cadherin antiserum (pAb120) as well as a series of monoclonal antibodies were raised against purified rat LI-cadherin from Morris Hepatom 7777 cells. The monoclonal anti-XB/U-cadherin antibody 6D5 was kindly provided by Dr. Peter Hausen (Max-Planck-Institute of Developmental Biology, Tübingen, Germany). Rabbit polyclonal antiserum (anti-CRD pAb) directed against the PI-PLC-digested form of the GPI-anchored *Leishmania* protein gp63 was a generous gift from Dr. Peter Overath (Max-Planck-Institute of Biology, Tübingen, Germany). The monoclonal anti- β -catenin antibody was purchased from Transduction Laboratories (Lexington, KY). FITC-conjugated phalloidin as well as all secondary antibodies used for immunoprecipitation and immunochemistry were from Sigma Chem. Co. (Deisenhofen, FRG). Peroxidase-conjugated secondary antibodies used for immunoblotting came from Dakopatts (Hamburg, FRG). The vital fluorescence membrane dye DiI (1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) was from Becton Dickinson (Heidelberg, FRG). [³H]Ethanolamine hydrochloride was obtained from Amersham Buchler GmbH (Braunschweig, FRG). All enzymes used in molecular biology methods were purchased from Pharmacia LKB Biotechnology (Freiburg, FRG). All other reagents were obtained from Sigma.

Cell Culture and Transfection

Parental mouse L cells (obtained from Amer. Type Culture Collection, Rockville, MD, No. CCL-1.3) were grown in DMEM supplemented with 10% FCS. Transfected cells were grown in the same medium in the presence of 0.2 mg/ml of G418 (Gibco BRL, Eggenstein, FRG). L cells were transfected with pRc/LIC by a modified calcium phosphate method. Briefly, 1 μg of the expression vector was precipitated and added to 0.5×10^6 cells grown on a 60-mm dish. After incubation for 5 h, cells were washed and were allowed to recover for 48 h in fresh medium. Transfected cells were selected in the presence of 1 mg/ml G418, and clones were established using cloning rings. Several LI-cadherin-expressing clones were isolated, and three clones, 12.1.10, 14.3.4, and 17.11.7, expressing approximately the same amount of LI-cadherin as assessed by Western blot analysis were used for subsequent experiments. For each of these clones identical results were obtained. Although the cells were truly clonal, expression of LI-cadherin in all isolated clones was unstable and LI-cadherin-negative cells appeared after several passages. To obtain a large number of cells expressing LI-cadherin at the same level, fluorescence activated cell sorting was used. For each separation, $\sim 1.0 \times 10^7$ cells were washed with PBS, detached with 2 mM EDTA in PBS containing 2% chicken serum, harvested by centrifugation, and resuspended in 1 ml of a 1:2-dilution of DMEM, 8% FCS in PBS (DMEM/PBS). Cells were incubated with 40 $\mu\text{g}/\text{ml}$ anti-LI-cadherin pAb120 for 60 min at 4°C. After washing in DMEM/PBS, cells were resuspended in 1 ml of the same buffer supplemented with FITC-conjugated goat anti-rabbit antibodies (Sigma) and incubated for 45 min at 4°C in dark. Cells were then washed three times in PBS, resuspended in 1 ml FCS-free DMEM, and kept on ice until being separated on a FACS Vantage™ System (Becton Dickinson). As a control, cells were incubated with DMEM/PBS followed by an incubation with the same FITC-labeled secondary antibodies. Cells were gated using forward versus side scatter to exclude dead cells and debris. Only those cells showing the highest expression levels of LI-cadherin ($\sim 10\%$ of the total population) were isolated and plated directly on glass coverslips in 24-well plates. L cells expressing *Xenopus* XB/U-cadherin were generated as described elsewhere (Kühl et al., 1996).

Drosophila (S2) cells (Schneider, 1972) were grown in revised Schneider's medium (Gibco BRL) supplemented with 12.5% FCS (Sigma). Cells were

1. Abbreviations used in this paper: CRD, cross-reacting determinant; GPI, glycosyl phosphatidylinositol; PCMBs, *p*-chloromercuriphenylsulfonic acid; PI-PLC, phosphatidylinositol-specific phospholipase C.

maintained at 25°C with air as the gas phase. For transfection, the expression vectors pRmHa-LI or pRmLI^{GPI} were mixed at a molecular ratio of 10:1 with pPC4, a plasmid conferring α -amanitin resistance as the selectable marker (Jokerst et al., 1989), and coprecipitated with calcium phosphate according to Sambrook et al. (1989). Cells (10^7 in a 60-mm-dish) were incubated overnight with the precipitate, washed, and were allowed to recover for 72 h in fresh medium. After 3 wk of selection in medium containing 5 μ g/ml α -amanitin (Sigma), transfected cells were cloned in 0.3% soft agar as described previously (Berndorff et al., 1994). Individual clones were induced with 0.7 mM CuSO₄ for 2–3 d and were assayed by Western blotting for high protein expression. The clones with highest expression of LI-cadherin or LI-cadherin^{GPI} were designated S2/LI-cad and S2/LI-cad^{GPI}, respectively, and were used for all subsequent experiments.

Construction of cDNA Expression Vectors

Full-length cDNA of rat LI-cadherin was excised from plasmid pTB2 (Berndorff et al., 1994) by digestion with NotI and partial digestion with ApaI, and was inserted into NotI/ApaI-restricted pRc/CMV (Invitrogen, NV Leek, NL). The resultant plasmid was designated pRc/LIC.

For the construction of a GPI-anchored form of LI-cadherin, a 2.5-kb cDNA fragment encoding the first 789 amino acids of LI-cadherin was isolated from pRmHa-LI (Berndorff et al., 1994) by digestion with KpnI and partial digestion with AccI. A DNA fragment encoding the *Drosophila* fasciclin I GPI anchor signal (Zinn et al., 1988) was adapted by PCR from a fasciclin I cDNA in pBluescript SK/+ using primer I (5'-CAACG-TATACGGCCCCGATGTTG-3') and primer II (5'-GCGGATCCG-GATTTGTTTTACATATCGG-3'). Primer I is identical to the coding strand of the fasciclin I cDNA (nucleotides 1967–89) but causes the deletion of one nucleotide to adopt the correct reading frame. Primer II introduces the underlined BamHI restriction site at the 3' end of the PCR product. The PCR product was digested with AccI and BamHI and was ligated in tandem with the 2.5-kb KpnI/Acc I-fragment of pRmHa-LI into KpnI/BamHI-restricted pRmHa-3. The correct ligation product, the plasmid pRmLI^{GPI}, was verified by DNA sequencing of both strands using the dideoxy method (Sanger et al., 1977).

SDS-PAGE and Western Blotting

SDS-PAGE was performed according to Laemmli (1970) and proteins were electrophoretically transferred to HybondTM-C membranes (Amersham Buchler GmbH, Braunschweig, FRG). Membranes were blocked for 1 h in TBST (25 mM Tris/HCl, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20) containing 5% nonfat dry milk, incubated for 1 h each with primary antibody and the appropriate peroxidase-conjugated secondary antibody (both in TBST, 5% nonfat dry milk), and were developed with the ECL detection system (Amersham). For reprobing with another antibody, membranes were stripped overnight at 42°C in 65 mM Tris/HCl, pH 6.6, containing 2% SDS and 100 mM β -mercaptoethanol, washed thoroughly with TBST, blocked, and processed as described above.

Immunocytochemistry

L cells were grown to confluency on glass coverslips, fixed in a fresh solution of PLP (26 mM Na-phosphate, pH 7.4, 10 mM NaIO₄, 94 mM lysine, 2% paraformaldehyde) for 20 min at room temperature and rinsed in PBS containing 0.1 M glycine. For the staining of cytoplasmic proteins, cells were permeabilized for 5 min with 0.2% Triton X-100 in PBS. After washing with PBS, the cells were incubated for 30 min in blocking buffer (PBS, 1% FCS, 1% BSA). Incubation with primary antibody was in blocking buffer for 1 h, followed by washing and incubation with fluorophore-conjugated secondary antibody (in blocking buffer) for 1 h. After washing, cells were mounted in Elvanol and examined using a Zeiss Axiophot fluorescence microscope. For detergent extraction, cells were preincubated for 5 min at 4°C in PBS containing 5% NP-40, washed in PBS, and processed as described above.

Immunofluorescence microscopy of S2 cells was performed as described previously (Berndorff et al., 1994). Briefly, cells were harvested after aggregation, washed twice with TBS/C (25 mM Tris/HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 2 mM CaCl₂) and fixed at room temperature for 15 min in TBS/C containing 3.5% formaldehyde. Fixed cells were washed and stained with FITC-labeled anti-LI-cadherin pAb120 for 1 h. The cells were finally resuspended in fluorescence buffer (885 mM Tris/HCl, pH 8.0, 0.5% *n*-propyl gallate, 10% glycerol) and mounted on slides.

Cell Adhesion Assays

Aggregation assays with L cells were performed as described previously (Ozawa et al., 1990). Briefly, cells were washed and treated with 0.01% trypsin in HBS (10 mM Hepes, pH 7.4, 37 mM NaCl, 5.4 mM KCl, 0.34 mM Na₂HPO₄, 5.6 mM glucose) containing 2 mM CaCl₂ for 10 min at 37°C. After washing in a 1:2 dilution of DMEM (containing 4% FCS) in HBS, cells were resuspended in the same buffer supplemented with 5 μ g/ml DNase I. The single cell suspension (5.0×10^5 cells in 500 μ l) was allowed to aggregate for 30 min at room temperature in 24-well plates on a rotary shaker (80 rpm). Aggregation was either performed in buffer without additive, or in buffer supplemented with 2 mM EDTA or with anti-LI-cadherin pAb120. To disrupt the cytoskeleton before the aggregation assay, cells were preincubated with 1 μ M cytochalasin D for 30 min at 37°C.

Transfected S2 cells were induced with 0.7 mM CuSO₄ for 2 d at 25°C, collected by centrifugation and resuspended in Schneider's medium to a density of 1.0×10^6 cell/ml. Cells were gently dissociated by pipetting and 500 μ l of the single cell suspension were agitated at room temperature for 1 h in 24-well plates on a rotary shaker (80 rpm). Aggregation assays were performed in Schneider's medium (containing 5 mM CaCl₂) or in the same medium supplemented with either 30 mM EDTA or with anti-LI-cadherin pAb120. For pretreatment with PI-PLC, the cell suspension (5.0×10^5 cells in 250 μ l) was incubated with 1 U/ml PI-PLC from *B. thuringiensis* for 2 h at 37°C. Subsequently the cell suspension was diluted to 1.0×10^6 cells/ml with medium and the extent of aggregation was measured as described previously (Berndorff et al., 1994).

In cell mixing experiments, one cell line was labeled in vivo by adding 1% (vol/vol) of the fluorescent membrane dye DII (0.5 mM stock solution in ethanol) to the cell suspension. After incubation for 15 min at 37°C, excess dye was removed by washing the cells twice in PBS. Cells were resuspended in medium to a density of 1.0×10^6 cells/ml, mixed with unlabeled cells, induced, and agitated on a rotary shaker (80 rpm) for 16 h at room temperature.

Isolation of Membrane Proteins

S2 cells were induced as described and harvested by centrifugation. About 2×10^7 cells were resuspended in 1 ml TBS/C containing 2% protease inhibitor mix (1 mg/ml leupeptin, pepstatin A, and chymostatin, each), as well as 5 mM *p*-chloromercuriphenylsulfonic acid (PCMBs) to inhibit any endogenously expressed PI-PLC activity in S2 cells. Cellular membranes were prepared as described (Hortsch, 1994) and their protein content was determined using the BCA protein assay (Pierce, Rockford, IL).

Treatment with PI-specific Phospholipase C

To remove trace amounts of PCMBs before PI-PLC digestion, cellular membranes containing 100 μ g protein were washed twice with TBS, pH 7.4, and resuspended in 49 μ l TBS, pH 7.4 containing 2 mM DTT, 2.5 mM EDTA, 0.2% Triton X-100 and 2% protease inhibitor mix. After addition of 1 μ l of PI-PLC from *T. Brucei* (generously provided by Dr. Peter Overath, Max-Planck-Institute of Biology, Tübingen, Germany), samples were incubated for 4 h at room temperature, mixed with 50 μ l 2% SDS sample buffer and boiled for 5 min. Proteins were separated by SDS-PAGE and subjected to Western blot analysis as described.

Metabolic Labeling and Immunoprecipitation

To collect radiolabeled immunoprecipitates, L cells were incubated with 5 MBq TRAN³⁵S-label (ICN Biomedicals GmbH, Eschwege, FRG) in methionine-free MEM (Gibco BRL) for 16 h. Cells were washed and lysed in 500 μ l extraction buffer (0.5% NP-40, 0.5% Triton X-100, 2 mM PMSF, 2 mM CaCl₂, 2% protease inhibitor mix in TBS, pH 8.0) for 2 h at 4°C. Lysates were cleared by centrifugation and incubated with primary antibody (50 μ g pAb120 or 5 μ g 6D5) for 1 h. Immune complexes were incubated for 1 h with 50 μ l of a 10% protein A-Sepharose suspension. Beads were washed three times in washing buffer (50 mM Tris/HCl, pH 8.5, 500 mM NaCl, 1 mM MgCl₂, 2 mM CaCl₂, 0.05% NP-40 containing 1 mg/ml ovalbumin), and finally boiled in SDS sample buffer. The dissociated proteins were separated by SDS-PAGE and analyzed by fluorography using the Intensify Universal Autoradiography Enhancer (DuPont New England Nuclear[®], Bad Homburg, FRG).

For metabolic labeling of S2 cells, 10^7 cells were washed twice with TBS, pH 7.4, resuspended in 5 ml medium containing 100 μ Ci [³H]ethanolamine hydrochloride and induced with 0.7 mM CuSO₄. After an over-

night incubation, the cells were lysed and labeled proteins were analyzed by immunoprecipitation and fluorography as described above.

Results

Expression of LI-Cadherin in L Cells

Although the cytoplasmic domain of LI-cadherin does not exhibit any homology to that of classical cadherins, LI-cadherin was shown to mediate calcium-dependent cell-cell adhesion when transfected into *Drosophila* S2 cells (Berndorff et al., 1994). However, it has been unclear whether LI-cadherin like classical cadherins depends on interactions with the cytoskeleton via catenins or other cytoplasmic proteins to exert its adhesive function. To test this possibility, mouse L cells lacking endogenous cadherin activity were transfected with rat LI-cadherin cDNA using pRc/CMV. Transfected cells were cloned and monitored for the expression of LI-cadherin by immunoblotting with the polyclonal anti-LI-cadherin antibody pAb120 which was raised against purified rat LI-cadherin (Geßner, R., N. Loch, P. Bringmann, D. Berndorff, N. Schnoy, W. Reutter, and R. Tauber, manuscript in preparation). The antibody detected a protein which migrated as a broad double band of ~120 kD (Fig. 1 A) representing N-glycosylation variants of LI-cadherin as could be shown by PNGase F-digestion (not shown). No proteins were stained in nontransfected L cells (Fig. 1 A). To determine the distribution of LI-cadherin in transfected L cells, immunofluorescence staining using anti-LI-cadherin mAb 47.2 was performed. Although LI-cadherin was expressed on the cell surface and appeared concentrated at sites of cell-cell contact

(Fig. 1 B, b), the cells did not acquire the cobblestone-like appearance of L cells expressing classical XB/U-cadherin (Fig. 1 B, c). While nontransfected L cells showed the typical spindle-shaped morphology of fibroblasts (Fig. 1 B, d), expression of LI-cadherin induced a small change of this phenotype, resulting in extended regions of cell-cell contact (Fig. 1 B, e). L cells expressing XB/U-cadherin exhibited a rather epithelial phenotype and appeared tightly connected with cell-cell contacts being barely visible in phase contrast views (Fig. 1 B, f). In contrast, LI-cadherin-transfected cells never formed an entirely closed monolayer even when grown to confluency (Fig. 1 B, e).

LI-Cadherin Does Not Interact with Catenins or the Actin Cytoskeleton

To examine whether LI-cadherin is associated with catenins or other cytoplasmic components, immunoprecipitation from parental and transfected L cells was performed subsequent to metabolic labeling (Fig. 2). L cells expressing *Xenopus* XB/U-cadherin, a classical cadherin that has previously been shown to form complexes with catenins (Müller et al., 1994; Finnemann et al., 1995; Kühl et al., 1996), served as a control. Using anti-XB/U-cadherin monoclonal antibody 6D5, two proteins of 102 and 92 kD corresponding to α - and β -catenin could be coprecipitated with XB/U-cadherin (Fig. 2, lane 4). In contrast, no proteins were coprecipitated under the same conditions with LI-cadherin using anti-LI-cadherin pAb120 (Fig. 2, lane 2). It has been suggested that the introduction of catenin-binding sites into L cells, due to the transfection with classical cadherins, either induces the upregulation of expres-

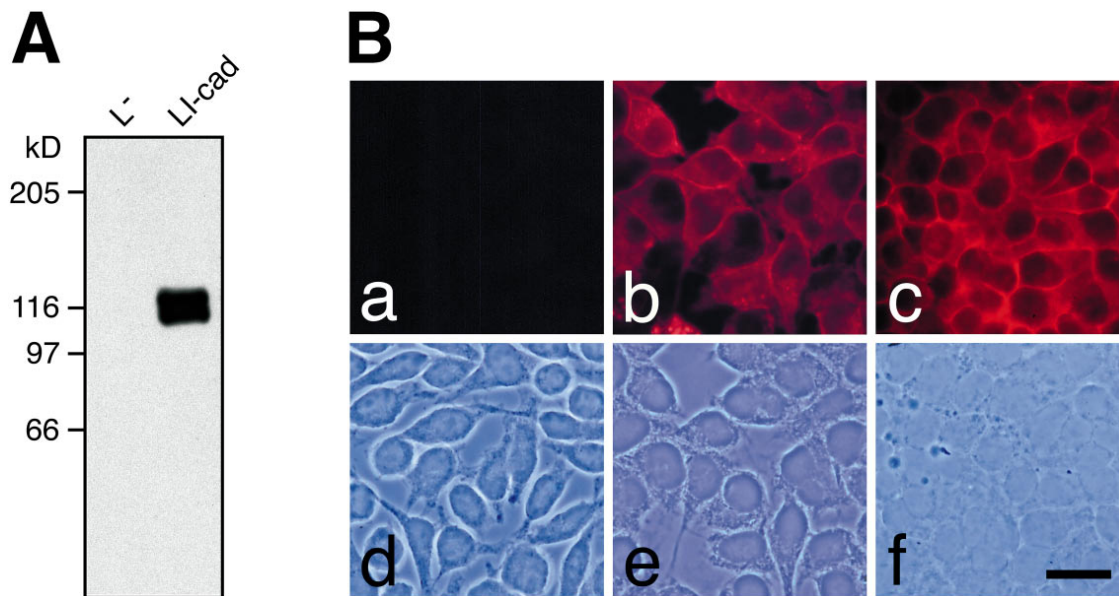


Figure 1. Expression of LI-cadherin in L cells. (A) Immunoblotting of LI-cadherin. Equal amounts (100 μ g) of proteins from parental (L⁻) and transfected L cells expressing LI-cadherin (LI-cad) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted using anti-LI-cadherin pAb120. LI-cadherin appeared as a broad double band with a molecular mass of ~120 kD. (B) Immunocytochemical staining of LI-cadherin. Parental (a and d) and transfected L cells expressing either LI-cadherin (b and e) or XB/U-cadherin (c and f) were fixed, incubated with anti-LI-cadherin mAb 47.2 (a and b) or anti-XB/U-cadherin mAb 6D5 (c), stained with TRITC-labeled secondary antibodies. The corresponding phase contrast micrographs are shown in panels d-f. LI-cadherin was expressed on the cell surface of transfected L cells and appeared concentrated at sites of cell-cell contact (b). In contrast to classical XB/U-cadherin (c and f), LI-cadherin did not induce an epithelial phenotype, although cell-cell contact regions were enlarged compared to nontransfected L cells (d and e). Bar, (f) 20 μ m.

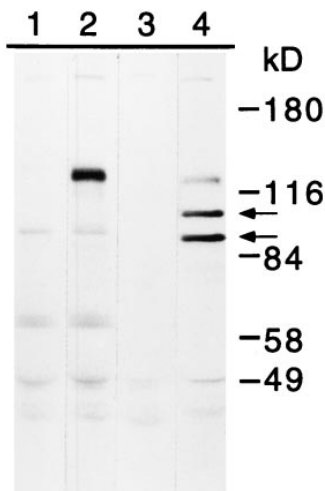


Figure 2. Catenins do not coprecipitate with LI-cadherin. Parental L cells (lanes 1 and 3) and transfected cells expressing either LI-cadherin (lane 2) or XB/U-cadherin (lane 4) were metabolically labeled, lysed, and subjected to immunoprecipitation using anti-LI-cadherin pAb120 (lanes 1 and 2) or anti-XB/U-cadherin mAb 6D5 (lanes 3 and 4). Precipitates were separated by SDS-PAGE and analyzed by autoradiography. While α - and β -catenin copurified with XB/U-cadherin (lane 4), neither catenins nor any other labeled proteins were coprecipitated with LI-cadherin (lane 2).

sion or leads to a reduced degradation of catenins (Nagafuchi et al., 1991, 1994; Shibamoto et al., 1995). Therefore, we determined whether the cellular concentration of β -catenin is influenced by the expression of LI-cadherin in transfected L cells. As shown in Fig. 3, the expression level of β -catenin in L cells remained unchanged after transfection with LI-cadherin cDNA (Fig. 3, lanes 4 and 5) while it was significantly elevated in cells expressing XB/U-cadherin (Fig. 3, lane 6). The combined results of both experiments indicate that LI-cadherin is not able to interact with catenins when expressed in L cells.

The complex formation with catenins is known to be a prerequisite for the interaction of classical cadherins with the cytoskeleton. Due to the catenin-mediated linkage to the cytoskeleton, intact cadherin molecules acquire a partial resistance to the extraction with non-ionic detergents (Nagafuchi and Takeichi, 1988; Ozawa et al., 1989). As shown in Fig. 4, *a* and *b*, LI-cadherin could not be detected in transfected L cells by immunofluorescence staining after pretreatment with NP-40. Under the same conditions XB/U-cadherin was only partially extracted and was still clearly detectable exhibiting a punctate staining pattern at cell-cell contact sites (Fig. 4, *c* and *d*). This indicates that LI-cadherin does not firmly interact with the cytoskeleton, which is consistent with the observation that LI-cadherin is unable to bind catenins (Figs. 2 and 3). This finding was confirmed by double fluorescence labeling of actin and either LI-cadherin or XB/U-cadherin in transfected L cells. In L cells expressing XB/U-cadherin, the actin cytoskeleton was completely redistributed to cell-cell contacts resulting in almost identical staining patterns of actin and XB/U-cadherin (Fig. 5, *c* and *d*). In contrast, the actin distribution in LI-cadherin-transfected cells remained unchanged and stress fibers were still present (Fig. 5, *a* and *b*). In summary, these results demonstrate that LI-cadherin is neither stably connected to the actin cytoskeleton, nor able to promote its reorganization.

LI-Cadherin-mediated Cell-Cell Adhesion Does Not Depend on an Intact Actin Cytoskeleton

The adhesive function of classical cadherins is dependent

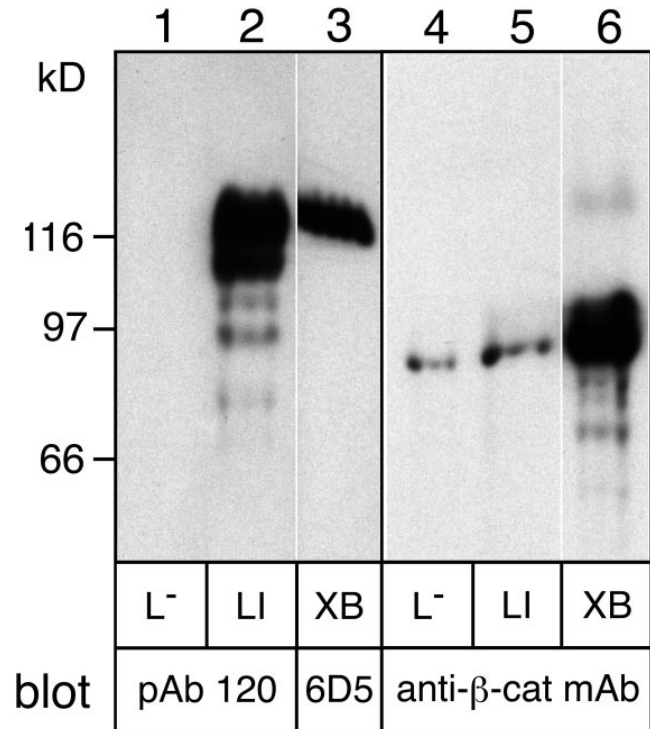


Figure 3. β -Catenin expression is not upregulated in LI-cadherin-transfected L cells. Equal amounts (100 μ g) of proteins from parental (lanes 1 and 4) and transfected L cells expressing either LI-cadherin (lanes 2 and 5) or XB/U-cadherin (lanes 3 and 6) were separated by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted using anti-LI-cadherin pAb120 (lanes 1 and 2), anti-XB/U-cadherin mAb 6D5 (lane 3) or anti- β -catenin mAb (lanes 4-6). Although LI-cadherin was highly expressed in transfected L cells (lane 2), the β -catenin expression remained the same as in nontransfected cells (lanes 4 and 5). In contrast, expression of XB/U-cadherin (lane 3) induced a significant increase of β -catenin in L cells (lane 6).

on the complex formation with catenins resulting in stable linkage to the cytoskeleton. Mutant cadherin molecules with deletions in their catenin-binding site fail to induce cell aggregation of transfected L cells (Nagafuchi and Takeichi, 1988, 1989; Ozawa et al., 1990). Since LI-cadherin is not stably connected to the actin cytoskeleton in transfected L cells, we examined whether it is nevertheless capable of inducing cell aggregation. Single cell suspensions of LI-cadherin-expressing L cells were incubated for 30 min on a rotary shaker and monitored by phase contrast microscopy for aggregation. In the presence of Ca^{2+} the cells formed aggregates containing ~ 50 -100 cells (Fig. 6 *a*). Aggregation could be completely inhibited by the removal of Ca^{2+} with EDTA or by incubation with anti-LI-cadherin pAb120 (Fig. 6, *b* and *c*). However, disruption of the actin-based cytoskeleton by preincubation with cytochalasin D had no effect on LI-cadherin-mediated cell aggregation (Fig. 6 *d*), whereas XB/U-cadherin-expressing cells remained disperse under these conditions (not shown). These results demonstrate that LI-cadherin is a functional Ca^{2+} -dependent cell adhesion molecule when expressed in L cells.

The finding that the adhesive function of LI-cadherin is

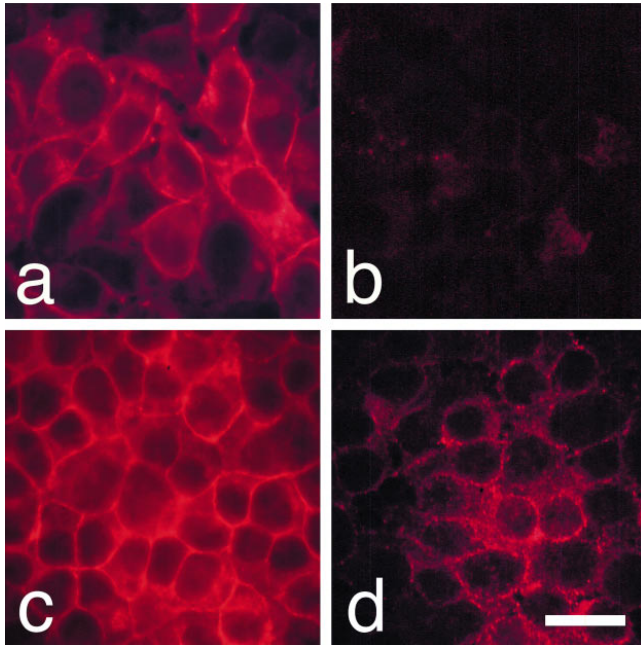


Figure 4. LI-cadherin is not resistant to extraction with NP-40. L cells expressing either LI-cadherin (*a* and *b*) or XB/U-cadherin (*c* and *d*) were fixed before (*a* and *c*) or after (*b* and *d*) extraction with 0.5% NP-40. Immunofluorescence staining was performed using anti-LI-cadherin mAb 47.2 (*a* and *b*) or anti-XB/U-cadherin mAb 6D5 (*c* and *d*) followed by an incubation with secondary TRITC-labeled antibodies. As shown in *b*, LI-cadherin could be easily extracted with NP-40, while XB/U-cadherin was partially resistant and remained clearly visible at cell-cell contacts under these conditions (*d*). Bar, (*d*) 20 μ m.

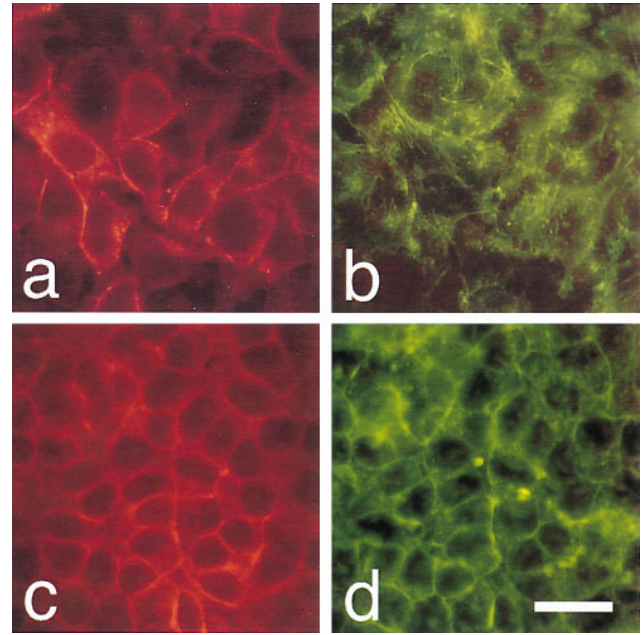


Figure 5. Actin cytoskeleton reorganization is not induced by LI-cadherin expression. L cells expressing either LI-cadherin (*a* and *b*) or XB/U-cadherin (*c* and *d*) were fixed, permeabilized, and the actin cytoskeleton was stained with FITC-phalloidin (*b* and *d*). For double labeling, the same cells were incubated with anti-LI-cadherin mAb 47.2 (*a*) or anti-XB/U-cadherin mAb 6D5 (*c*) followed by staining with secondary TRITC-labeled antibodies. In transfected L cells expressing XB/U-cadherin, the actin cytoskeleton was completely redistributed to sites of cell-cell contact (*d*). In contrast, expression of LI-cadherin did not promote any significant reorganization of the actin cytoskeleton and stress fibers were still visible (*b*). Bar, (*d*) 20 μ m.

independent of catenin binding and the subsequent linkage to the cytoskeleton clearly distinguishes this molecule from classical cadherins. Nevertheless, it is unclear whether the ability to mediate cell-cell adhesion is brought about solely by the enlarged extracellular domain of LI-cadherin or whether it is dependent on its transmembrane and cytoplasmic domain. To discriminate between these possibilities, a chimeric protein was constructed, in which the transmembrane and the cytoplasmic domain of LI-cadherin have been replaced by a GPI anchor signal sequence.

Construction of GPI-anchored LI-Cadherin^{GPI}

An artificial GPI-anchored form of LI-cadherin (LI-cadherin^{GPI}) was generated, thus excluding any direct interaction of the mutant protein with cytoplasmic components (Fig. 7). In the fusion protein the extracellular domain of LI-cadherin is linked directly to the GPI anchor signal sequence of fasciclin I, a homophilic neural cell adhesion molecule expressed on a subset of fasciculating axons in both, the grasshopper and the *Drosophila* embryo (Zinn et al., 1988; Elkins et al., 1990; Hortsch and Goodman, 1990). When processed correctly, LI-cadherin^{GPI} should contain the complete extracellular domain of LI-cadherin, followed by the last 28 amino acids of mature fasciclin I and the carboxyterminally linked GPI-anchor. Since the domains responsible for the adhesive function of fasciclin I

are located near the amino terminus (Seeger, M., personal communication), it can be ruled out that the small carboxy-terminal fasciclin I-derived portion does contribute to the adhesive properties of the fusion protein.

Native and GPI-anchored LI-cadherin were expressed in *Drosophila* S2 cells (Schneider, 1972) which are capable to correctly process the fasciclin I GPI anchor signal (Hortsch et al., 1995). Moreover, S2 cells exhibit a non-adherent phenotype and have previously been shown to constitute an excellent tool for the functional analysis of vertebrate cell adhesion molecules (Berndorff et al., 1994; Felsenfeld et al., 1994). The cDNAs encoding either LI-cadherin or LI-cadherin^{GPI} were introduced into S2 cells using the pRmHa-3 vector in which cDNA expression is driven by an inducible *Drosophila* metallothionein promoter (Bunch et al., 1988). Transfected cells were cloned in soft agar and selected for high expression levels of LI-cadherin or LI-cadherin^{GPI}. The resulting cell lines were designated S2/LI-cad and S2/LI-cad^{GPI}.

LI-Cadherin^{GPI} Is Expressed as a GPI-anchored Integral Membrane Protein in S2 Cells

To examine whether LI-cadherin^{GPI} is correctly processed and bound to the plasma membrane via a GPI anchor, detergent-treated membrane fractions from parental and transfected S2 cells were incubated with PI-specific phos-

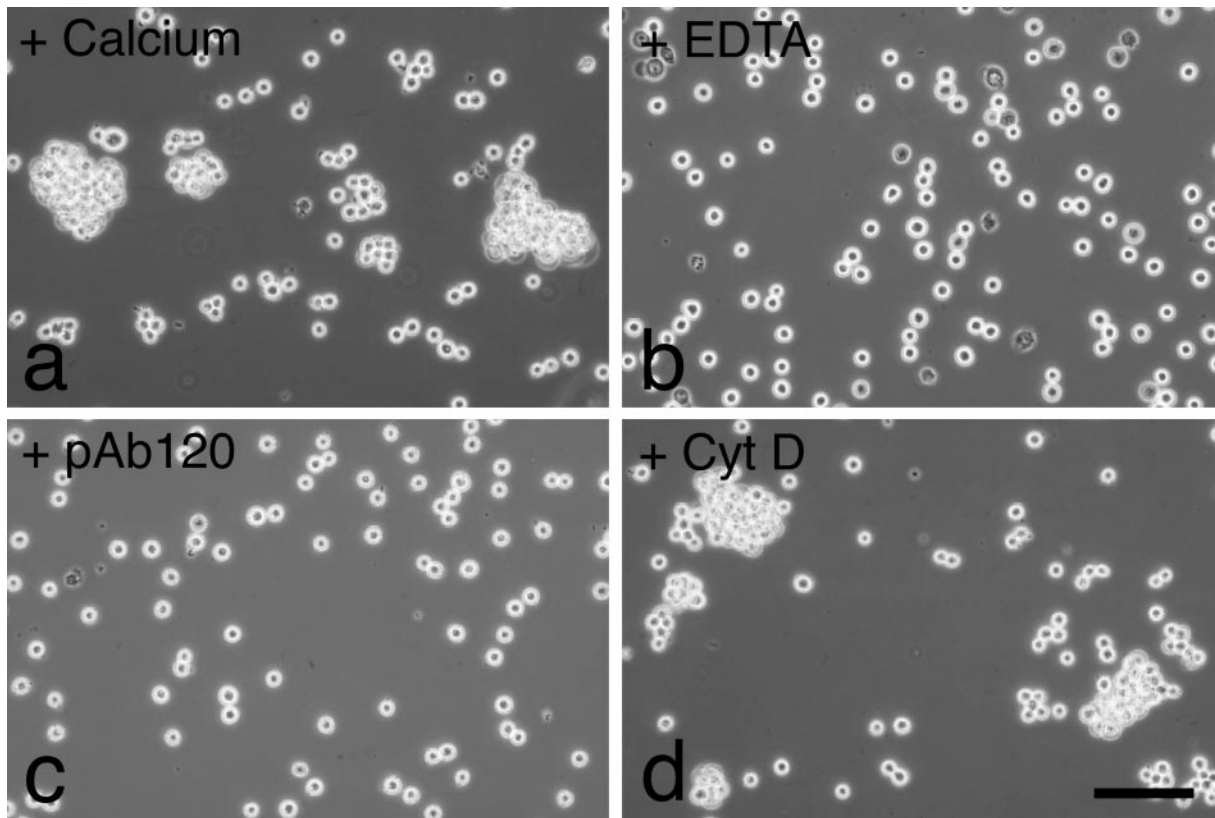


Figure 6. LI-cadherin mediates aggregation of transfected L cells. Aggregation of LI-cadherin expressing L cells was analyzed in the presence of 2 mM CaCl_2 (a), 2 mM EDTA (b) or anti-LI-cadherin pAb120 (c). For the disruption of the cytoskeleton (d), cells were preincubated with 1 μM cytochalasin D for 30 min at 37°C. LI-cadherin acted as a Ca^{2+} -dependent cell adhesion molecule when expressed in L cells. Its function was not affected by the disruption of the actin cytoskeleton.

pholipase C (PI-PLC) from *T. Brucei*, separated by SDS-PAGE, and analyzed by immunoblotting. Staining with anti-LI-cadherin pAb120 showed that LI-cadherin and LI-cadherin^{GPI} were expressed in similar amounts by the clonal cell lines S2/LI-cad and S2/LI-cad^{GPI} (Fig. 8 A, lanes 3 and 5). Both proteins have an apparent molecular mass of ~110 kD which was not significantly changed upon PI-PLC treatment (Fig. 8 A, lanes 3–6). No immunoreactive proteins were found in membranes of untransfected S2 cells (Fig. 8 A, lanes 1 and 2). The blot was stripped and reprobed with a polyclonal antibody (anti-CRD pAb) against the cross-reacting determinant of GPI anchors, an epitope which is exposed in GPI-anchored molecules solely after digestion with PI-PLC (Zamze et al., 1988). CRD-specific antibodies were unable to detect any membrane proteins produced by either untransfected or S2/LI-cad cells, irrespective of PI-PLC treatment (Fig. 8 A, lanes 7–10). Likewise, undigested membranes from S2/LI-cad^{GPI} cells did not contain any immunoreactive proteins (Fig. 8 A, lane 11). However, after incubation with PI-PLC, a single protein band was detected in these membranes at ~110 kD (Fig. 8 A, lane 12), indicating that LI-cadherin^{GPI} is correctly processed in *Drosophila* S2 cells and is recognized as a substrate by PI-specific PLC.

Since ethanolamine is an integral part of the GPI anchor (Fig. 7), metabolic labeling with [³H]ethanolamine can be used to identify GPI-anchored proteins (Cross, 1990). To

verify independently the correct processing of LI-cadherin^{GPI}, immunoprecipitation using anti-LI-cad pAb120 was performed after metabolic labeling of parental and transfected S2 cells with [³H]ethanolamine. In extracts of S2/LI-cad^{GPI} cells a 110-kD protein was found to be metabolically labeled with [³H]ethanolamine (Fig. 8 B, lane 3). This protein could be specifically immunoprecipitated with anti-LI-cadherin pAb120 (Fig. 8 B, lane 6) demonstrating that the [³H]ethanolamine moiety has been covalently incorporated into the GPI anchor of LI-cadherin^{GPI}. Since unmodified LI-cadherin could not be labeled with [³H]ethanolamine (Fig. 8 B, lanes 2 and 5), the modification itself must be solely responsible for the change. Any unspecific binding of [³H]ethanolamine to the extracellular domain of LI-cadherin can be ruled out, since this domain is identical in both proteins.

Taken together these experiments demonstrate that LI-cadherin^{GPI} is correctly processed and expressed in S2 cells. It is attached to the plasma membrane via an intact GPI anchor that is susceptible to cleavage by PI-PLC.

The Adhesive Function Is Preserved in LI-Cadherin^{GPI}

To quantitatively compare the cell adhesion activity of native and GPI-anchored LI-cadherin, a cell adhesion assay was performed, and aggregation was calculated as percent reduction in particle number over an incubation period of

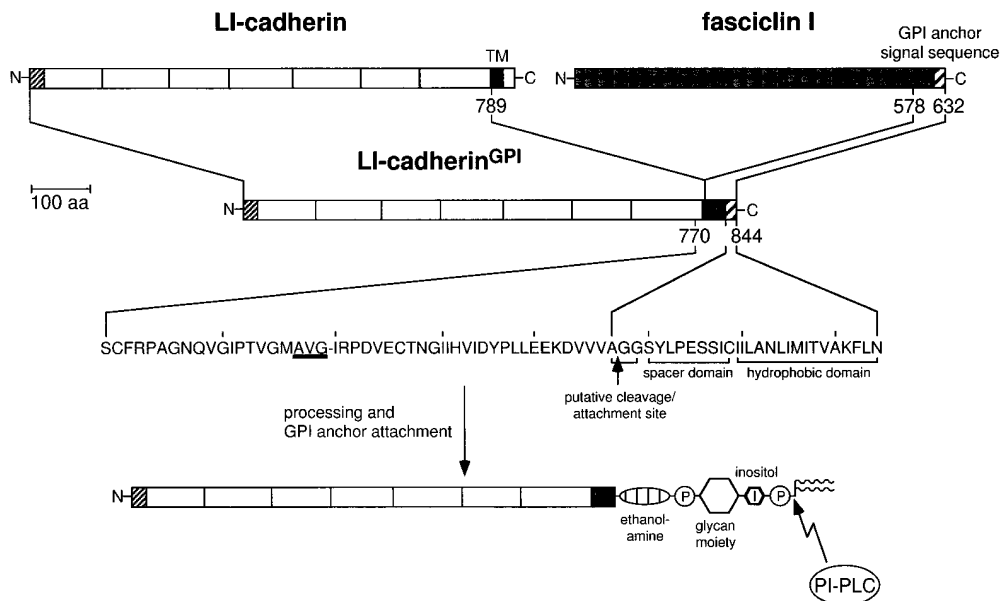


Figure 7. Construction of GPI-anchored LI-cadherin^{GPI}. The first 789 amino acids representing the entire extracellular domain of LI-cadherin were linked to the COOH-terminal 55 amino acids of *Drosophila* fasciclin I. The fasciclin I-derived portion of LI-cadherin^{GPI} contains a typical signal sequence for GPI anchoring (Coyne et al., 1993; Kodukula et al., 1993). This signal sequence comprises a domain with small amino acids at the first position (representing the putative cleavage/GPI anchor attachment site), and at the third position, followed by a 9-amino acid-spacer domain and a hydrophobic region of 16 amino acids. After cleavage

of the signal peptide in the ER, the GPI anchor is linked to the new COOH terminus of the protein via an ethanolamine residue (for reviews see Cross, 1990; Englund, 1993). The last LI-cadherin-derived amino acids (AVG) are underlined in the protein sequence of LI-cadherin^{GPI}.

60 min. In the presence of Ca²⁺, LI-cadherin^{GPI} mediated cell-cell adhesion to the same extent as wild-type LI-cadherin (Fig. 9, Ca²⁺). Under these conditions, no significant aggregation of untransfected S2 cells was observed (Fig. 9, control). Addition of EDTA or anti-LI-cadherin pAb120 entirely inhibited the aggregation of both S2/LI-cad and S2/LI-cad^{GPI} cells. The complete inhibition of LI-cadherin^{GPI}-mediated cell-cell adhesion by anti-LI-cadherin antibodies and its strict Ca²⁺ dependence rules out that the fasciclin I-derived portion of the fusion protein is contributing to its adhesive function.

Furthermore, preincubation with PI-PLC inhibited the aggregation of S2/LI-cad^{GPI} cells to a similar extent as did addition of EDTA or anti-LI-cadherin pAb120, while the aggregation of S2/LI-cad cells remained unchanged (Fig. 9, PI-PLC). These results demonstrate that native and GPI-anchored LI-cadherin are indistinguishable in their ability to mediate Ca²⁺-dependent cell-cell adhesion. However, this activity is completely abolished by PI-PLC digestion, indicating that the adhesive function of LI-cadherin^{GPI} is dependent on an intact GPI anchor. To examine the distribution of LI-cadherin^{GPI} within aggregates of S2/LI-cad^{GPI} cells, immunofluorescence staining with FITC-labeled anti-LI-cadherin pAb120 was carried out (Fig. 10). LI-cadherin^{GPI} was expressed all over the cell surface including those regions that are not in direct contact with neighboring cells. However, an increased staining was observed at sites of cell-cell contact (Fig. 10), which is consistent with the notion that LI-cadherin^{GPI} is a functional cell adhesion molecule. Interestingly, LI-cadherin^{GPI} did not appear in clusters on the cell surface, which is in contrast to the clustering that has been frequently observed in other cells for GPI-anchored molecules (reviewed by Anderson, 1993) including the only naturally occurring GPI-anchored cadherin, T-cadherin (Vestal and Ranscht, 1992).

LI-Cadherin^{GPI} Induces Aggregation in a Homophilic Manner and Interacts with Native LI-Cadherin

Cadherin-mediated cell-cell adhesion is caused by the homophilic binding of identical cadherin molecules on the surface of adjacent cells (Takeichi, 1991). For this reason, cell mixing experiments were performed, to determine whether the binding specificity of LI-cadherin^{GPI} differs from that of native LI-cadherin due to its altered type of membrane insertion. Parental S2 cells were labeled with the fluorescent membrane dye DiI, mixed with unlabeled S2/LI-cad^{GPI} cells, and assayed for aggregation. Fig. 11 shows that untransfected S2 cells remained disperse and were excluded from aggregates formed by cells expressing LI-cadherin^{GPI} (Fig. 11, *a* and *b*). In a second mixing experiment, S2/LI-cad cells were labeled and aggregated together with unlabeled S2/LI-cad^{GPI} cells. Large aggregates were formed that contained both labeled and unlabeled cells in a random distribution (Fig. 11, *c* and *d*).

These findings demonstrate that the observed cell aggregation is a result of homophilic LI-cadherin^{GPI}-mediated cell-cell adhesion, and is not due to a heterophilic interaction between LI-cadherin^{GPI} and a potential endogenous receptor expressed by S2 cells. Furthermore, the binding specificity of LI-cadherin^{GPI} seems to be unaffected by the deletion of the transmembrane and cytoplasmic domains.

Discussion

LI-cadherin is a novel member of the cadherin superfamily exhibiting an unusual protein structure compared to classical cadherins. One unique feature of LI-cadherin is the small size of its cytoplasmic domain. This domain consists of only 20 amino acids and exhibits no homology to the corresponding region of classical cadherins which is es-

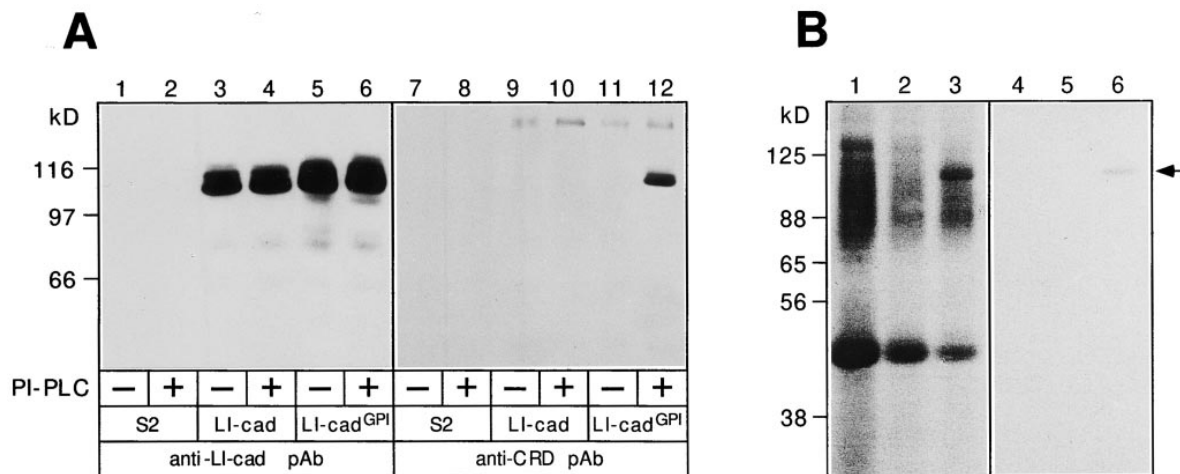


Figure 8. LI-cadherin^{GPI} is expressed as a GPI-anchored molecule in S2 cells. (A) Immunoblotting of LI-cadherin^{GPI}. Equivalent amounts (75 μ g) of membrane proteins from untransfected S2 cells (S2) and cells expressing either LI-cadherin (LI-cad) or LI-cadherin^{GPI} (LI-cad^{GPI}) were incubated with or without PI-PLC from *T. brucei*, separated by SDS-PAGE, and analyzed by immunoblotting using anti-LI-cadherin pAb120 (lanes 1–6). When expressed in S2 cells, both LI-cadherin and LI-cadherin^{GPI} exhibited an apparent molecular mass of \sim 110 kD, that was not changed detectably by PI-PLC treatment (lanes 4 and 6). The filter was stripped and reprobed with an antibody against the cross-reacting determinant (anti-CRD pAb) which is exposed solely in PI-PLC-cleaved GPI anchors (lanes 7–12). Only in cells expressing LI-cadherin^{GPI} a single 110-kD protein was stained by anti-CRD pAb after digestion with PI-PLC (lane 12). (B) Metabolic labeling of LI-cadherin^{GPI} with [³H]ethanolamine. After metabolic labeling with [³H]ethanolamine, untransfected S2 cells (lanes 1 and 4) and cells expressing either LI-cadherin (lanes 2 and 5) or LI-cadherin^{GPI} (lanes 3 and 6) were lysed and cellular extracts were subjected to immunoprecipitation using anti-LI-cadherin pAb120. Equivalent amounts (75 μ g) of solubilized proteins from the three cell types were separated in lanes 1–3. The corresponding immunoprecipitates are shown in lanes 4–6. The arrow indicates the position of immunoprecipitated LI-cadherin^{GPI} in lane 6 that has incorporated [³H]ethanolamine.

sential for their adhesive function (Takeichi, 1988, 1991; Geiger and Ayalon, 1992; Kemler, 1993). Nevertheless, LI-cadherin is capable of mediating Ca²⁺-dependent cell–cell adhesion when expressed in *Drosophila* S2 cells (Berndorff et al., 1994).

To examine whether the cytoplasmic domain is of similar importance for the adhesive function of LI-cadherin as it is for classical cadherins, we analyzed the interaction of LI-cadherin with cytoplasmic components in transfected L cells. In contrast to classical cadherins (Nagafuchi and Takeichi, 1989; Ozawa et al., 1989), no catenins or other copurified proteins were found in LI-cadherin immunoprecipitates from metabolically labeled cells. Furthermore, expression of LI-cadherin did not induce the upregulation of β -catenin expression observed for classical cadherins (Nagafuchi et al., 1991, 1994; Shibamoto et al., 1995). These observations demonstrate that the cytoplasmic domain of LI-cadherin is not associated with catenins. This can be explained by the lack of homology of this domain to the recently identified region of E-cadherin, which is essential for the interaction with catenins (Stappert and Kemler, 1994). It has been reported that nonfunctional cadherin molecules without catenin-binding activity can be easily extracted with nonionic detergents, while intact cadherins are resistant to this treatment due to their ability to interact with the cytoskeleton (Nagafuchi and Takeichi, 1988; Ozawa et al., 1989). We have found that LI-cadherin can be completely extracted with NP-40 under conditions where significant amounts of the classical XB/U-cadherin remain attached to the cytoskeleton. In addition, while classical cadherins colocalize with actin (Hirano et al., 1987; see

Fig. 5) and induce a redistribution of cytoskeletal proteins to the plasma membrane (McNeill et al., 1990), expression of LI-cadherin in transfected L cells did not result in a reorganization of the actin cytoskeleton. These results clearly demonstrate that LI-cadherin is not firmly attached to the actin cytoskeleton. This is consistent with the finding that the morphology of transfected L cells was only slightly changed due to the expression of LI-cadherin. Although sites of cell–cell contact were enlarged, LI-cadherin did not induce the epithelial phenotype adopted by L cells expressing classical cadherins. Despite these obvious differences, LI-cadherin was capable of mediating Ca²⁺-dependent cell–cell adhesion. In contrast to classical cadherins, however, adhesion by LI-cadherin was independent from an intact actin cytoskeleton.

There are two possible explanations for the ability of LI-cadherin to mediate cell–cell adhesion without binding to the cytoskeleton via catenins: One is based on a recently proposed model, in which the adhesive forces of individual cadherin molecules are bundled in a so-called “adhesion zipper” (Shapiro et al., 1995). Each element of the zipper is believed to consist of a cadherin dimer stabilized by hydrophobic interactions between adjacent cadherin molecules of one cell. In this respect it is conceivable that the lateral association of the aligned molecules is strengthened by the two additional cadherin-type repeats present in the extracellular domain of LI-cadherin. This stabilization may compensate for the missing intracellular linkage to the cytoskeleton, which induces the clustering of classical cadherins in adherens junctions. This hypothesis is subject of current investigations.

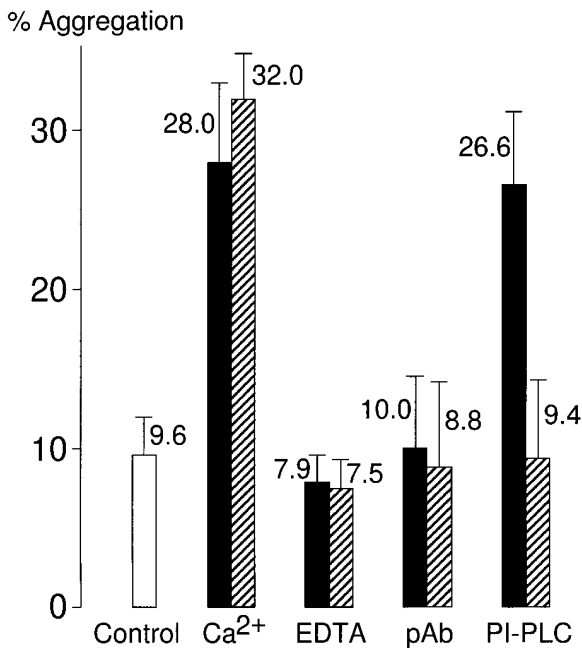


Figure 9. LI-cadherin^{GPI} is a functional cell adhesion molecule when expressed in S2 cells. Aggregation of transfected S2 cells expressing either LI-cadherin (black bars) or LI-cadherin^{GPI} (striped bars) was induced and quantified as percent reduction in particle number. Aggregation was carried out in Schneider's medium (containing 5 mM Ca²⁺), or after addition of either 30 mM EDTA or anti-LI-cadherin pAb120. For PI-PLC treatment, cells were incubated for 2 h with 1 U/ml PI-PLC from *B. thuringiensis* before aggregation. Untransfected S2 cells (white bar) in medium containing 5 mM Ca²⁺ served as an aggregation control. The column height corresponds to the mean of five aggregation experiments; the error bars indicate the standard deviation. LI-cadherin^{GPI} induced aggregation of transfected S2 cells in a Ca²⁺-dependent manner to the same extent as did wild-type LI-cadherin. Pretreatment with PI-PLC, and thus removal of LI-cadherin^{GPI} from the cell surface, caused a complete inhibition of aggregation.

The second possibility is that the clustering of LI-cadherin molecules is promoted by the interaction with auxiliary proteins, which bind to the short cytoplasmic domain of LI-cadherin but do not coprecipitate under standard conditions. This is conceivable, since other adhesion molecules containing only short intracellular domains have been reported to associate with cytoplasmic proteins, and thus become linked to the cytoskeleton. For example, the cytoplasmic 47 amino acids of β_1 integrin are able to bind α -actinin (Otey et al., 1990), as well as paxillin and pp125^{FAK} (Schaller et al., 1995). Furthermore, L-selectin has recently been shown to interact directly with α -actinin although its predicted cytoplasmic domain contains only 17 amino acids (Pavalko et al., 1995).

To test the second hypothesis, a GPI-anchored form of LI-cadherin (LI-cadherin^{GPI}) was constructed, thus excluding any interaction with cytoplasmic components. A similar approach has been used to demonstrate that the homophilic adhesive activity of *Drosophila* neuroglian is independent of its intracellular interaction with ankyrin (Hortsch et al., 1995). No interaction of a GPI-anchored neuroglian with the membrane cytoskeleton has been ob-

served (Dubreuil et al., 1996). In the present report the transmembrane and cytoplasmic domains of LI-cadherin were exchanged for the GPI anchor signal sequence of fasciclin I (Zinn et al., 1988). When expressed in *Drosophila* S2 cells, which have been used successfully for the functional analysis of both LI-cadherin (Berndorff et al., 1994) and fasciclin I (Elkins et al., 1990), LI-cadherin^{GPI} was correctly processed and linked to a GPI anchor. Despite the obvious lack of cytoplasmic interactions, LI-cadherin^{GPI} mediated Ca²⁺-dependent adhesion of transfected S2 cells to the same extent as wild-type LI-cadherin. Aggregation could be suppressed by calcium withdrawal, addition of LI-cadherin-specific pAb120 or preincubation with PI-PLC. Using cell mixing experiments we were able to show that cell-cell adhesion induced by LI-cadherin^{GPI} was homophilic, and that the binding specificity was not affected by the type of membrane attachment. Apparently, the adhesive function of LI-cadherin is independent of any interaction with cytoplasmic components. Thus, it can be concluded that the structure of the extracellular domain alone is capable to support the adhesive properties of LI-cadherin.

What are the physiological implications of a cadherin that mediates cell-cell adhesion without binding to the cytoskeleton? LI-cadherin is specifically expressed in liver and intestine, where it is found exclusively on the lateral surface of polarized cells outside of adherens junctions and desmosomes (Berndorff et al., 1994). In contrast, E-cadherin is found in the same cells preferentially in adherens junctions, but to some extent also on the basolateral surface (Boller et al., 1985). Enterocytes of the intestinal epithelium are derived from highly proliferative stem cells residing in the crypts of Lieberkühn and differentiate as they migrate into the villus region (Gordon, 1989). Interestingly, undifferentiated crypt cells from the adult chicken small intestine contain 15-fold higher levels of tyrosine phosphorylated proteins than do differentiated enterocytes (Burgess et al., 1989). Furthermore, a high level of pp60^{src} activity has been observed in dividing intestinal crypt cells, and the activity of this tyrosine kinase decreases during migration of enterocytes to the apex of the villus (Cartwright et al., 1993). Tyrosine phosphorylation of catenins by members of the src-family, which are found enriched in adherens junctions (Tsukita et al., 1991), is correlated with the inhibition of cell-cell adhesion mediated by classical cadherins (Matsuyoshi et al., 1992; Behrens et al., 1993; Hamaguchi et al., 1993), and with disintegration of adherens junctions (Volberg et al., 1992). Consequently, the adhesive function of E-cadherin should be reduced in undifferentiated enterocytes. Since LI-cadherin lacks cytoplasmic tyrosine residues and mediates cell-cell adhesion independent of catenin binding, its adhesive function should neither be affected by cadherin nor by catenin tyrosine phosphorylation. We thus propose a model in which the adhesive function of LI-cadherin is complementary to that of classical cadherins ensuring cell-cell adhesion throughout the entire enterocyte differentiation pathway even under conditions that cause downregulation of classical cadherins. This view is supported by the analysis of transgenic mice, which developed inflammatory bowel disease as a result of intestinal epithelial-specific expression of a mutated N-cadherin lacking the extracellular domain (Hermiston and

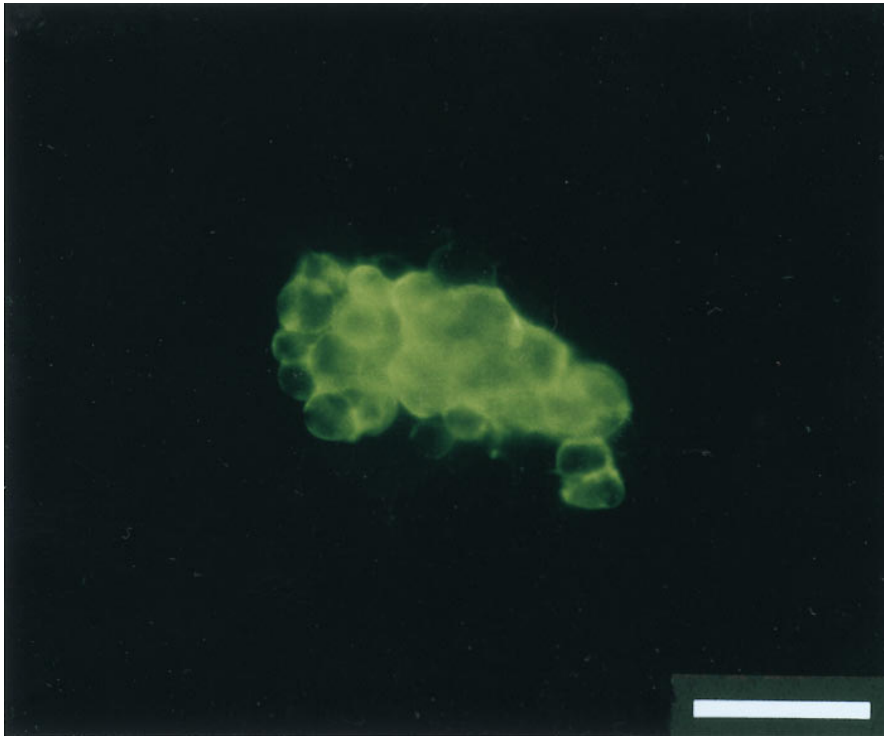


Figure 10. Surface expression pattern of LI-cadherin^{GPI}. The distribution of LI-cadherin^{GPI} within fixed aggregates of transfected S2 cells was determined by immunofluorescence staining with FITC-labeled anti-LI-cadherin pAb120. LI-cadherin^{GPI} was located at sites of cell-cell contact, but could also be found on cell surface regions that were not in direct contact with neighboring cells. Note that LI-cadherin^{GPI} did not appear in clusters on the cell surface. Bar, 50 μm .

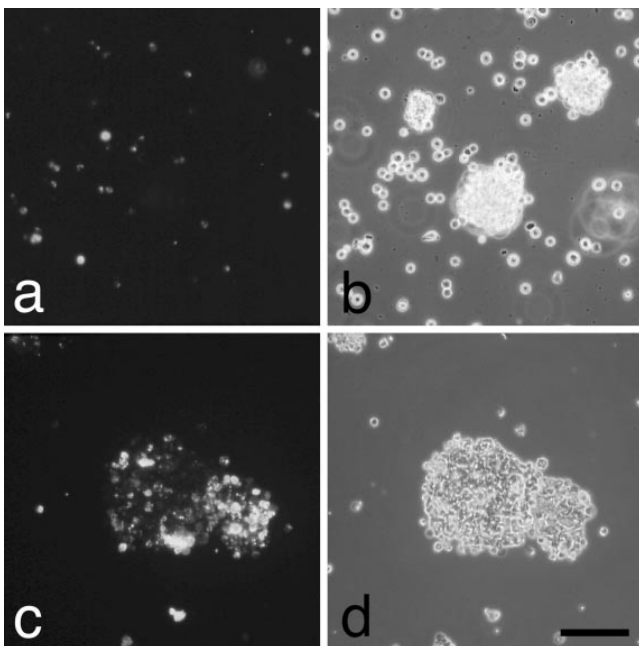


Figure 11. LI-cadherin^{GPI} is a homophilic cell adhesion molecule and is able to interact with native LI-cadherin. Parental S2 cells (*a* and *b*) or LI-cadherin-expressing S2 cells (*c* and *d*) were labeled with the vital fluorescence membrane dye DiI, mixed with unlabeled LI-cadherin^{GPI}-transfected cells and agitated together. Fluorescence micrographs are shown in *a* and *c*, the corresponding phase contrast micrographs are shown in *b* and *d*. LI-cadherin^{GPI} mediates cell-cell adhesion in a homotypic manner, since aggregates contained no DiI-labeled parental S2 cells (*a* and *b*). S2 cells expressing either native or GPI-anchored LI-cadherin form large mixed aggregates, suggesting that the adhesive properties of both proteins are indistinguishable (*c* and *d*). Bar, (*d*) 100 μm .

Gordon, 1995*a,b*). Despite the complete loss of E-cadherin function, the intestinal epithelium was only partially disrupted, and the enterocytes remained attached at their lateral sides.

Still, it cannot be excluded that LI-cadherin is able to laterally associate with yet unknown proteins. This consideration gains further support by the recent finding that LI-cadherin is the rat homologue (Böttinger, A., A. Volz, B. Kreft, C. Fieger, D. Patschan, N. Schnoy, R. Geßner, and R. Tauber, manuscript in preparation) of HPT-1, a protein involved in proton-dependent peptide transport across the intestinal epithelium (Dantzig et al., 1994). Moreover, a second cadherin with homologous structure, Ksp-cadherin, has been reported to be associated with a renal $\text{Na}^+/\text{HCO}_3^-$ cotransporter (Thomson et al., 1995). This opens the possibility that LI-cadherin, in addition to its adhesive function, might be associated with other transport proteins. Together with its apparent complementary function and its different extracellular structure, this clearly distinguishes LI-cadherin from GPI-anchored chicken T-cadherin (Ranscht and Dours-Zimmermann, 1991), the only other known cadherin that mediates Ca^{2+} -dependent cell-cell adhesion independent of interactions with the cytoskeleton (Vestal and Ranscht, 1992).

In summary, we were able to show that LI-cadherin is neither associated with catenins nor firmly linked to or dependent on an intact actin cytoskeleton. In sharp contrast to classical cadherins, cell-cell adhesion mediated by LI-cadherin is independent of any interaction with cytoplasmic components. We postulate that the adhesive function of LI-cadherin is complementary to that of coexpressed classical cadherins, and therefore may be important in the formation and maintenance of epithelial integrity in liver and intestine.

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