The Role of the Cell Adhesion Molecule Uvomorulin in the Formation and Maintenance of the Epithelial Junctional Complex

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Abstract. The role of the epithelial adhesion molecule uvomorulin in the formation of the epithelial junctional complex in the Madin-Darby canine kidney (MDCK) cell line was investigated. Experiments were carried out to determine whether specific inhibition of uvomorulin function would interfere selectively with the formation, stability, or function of the apical zonula adherens (ZA) and zonula occludens (ZO), or whether it would interfere with all forms of intercellular contact including the desmosomes. The effects of blocking antibodies and Fab fragments to uvomorulin on the formation of the junctional complex was examined with a Ca2+ switch assay for de novo junction assembly. The formation of the ZO, the ZA, and the desmosomes was assayed by fluorescence staining with an antibody to the tight junction-specific protein ZO-1, with rhodamine-phalloidin for ZA-associated actin filaments, and with an anti-desmoplakin antibody, respectively. Under different conditions and times of antibody treatment the extent of inhibition of the formation of each of the junctional elements was very similar. The ability of the cells to eventually overcome the inhibitory effect of the antibodies and form junctions correlated with the reappearance of uvomorulin at the regions of cell-cell contact. Therefore uvomorulin seems to mediate an early adhesion event between epithelial cells that is a prerequisite for the assembly of all elements of the junctional complex. In contrast, the transepithelial electrical resistance of confluent, well-established monolayers of MDCK cells grown on filters was not greatly affected by treatment with the various antibodies or Fab fragments. A small transient decrease in resistance observed with the polyclonal α-uvomorulin IgG may be due to a more subtle modulation of the junctional complex.

The cell surface glycoprotein uvomorulin plays an important role in the formation of cell-cell contacts in epithelial tissues (2, 21, 24). Uvomorulin is also known as E-cadherin (36, 40), and is very likely the same molecule as L-CAM (3, 11, 16) and cell-CAM 120/80 (9) in other species. It mediates the Ca2+-dependent process of compaction in the early mouse embryo (9, 24, 40) and the Ca2+-dependent aggregation of isolated epithelial cells (3, 30, 40).

Epithelial cells are also connected together by the epithelial junctional complex, which consists of the zonula occludens (ZO) or tight junction, the zonula adherens (ZA) or intermediate junction, and the desmosomes (12). The formation and stability of these three elements of the junctional complex are also dependent on extracellular Ca2+ (17, 19, 20, 22, 28, 31, 39).

The relationship between cell adhesion and cell junction formation has not been clearly elucidated. One prominent hypothesis in the literature is that cell adhesion is an early recognition event between cells that is a prerequisite to the formation of specialized intercellular junctions (11, 36). Yet, desmosomes and the ZA probably also play important roles in intercellular adhesion (25, 39). In fact, the distinction between cell adhesion molecules and intercellular junctional molecules has become blurred recently. In many epithelial cell types uvomorulin has been found to be highly concentrated in the ZA (2, 4, 38). Also, Gumbiner and Simons (21) found that uvomorulin was critically involved in the rapid rescaling of tight junctions between confluent Madin–Darby canine kidney (MDCK) cells. From these findings they proposed the alternative hypothesis that uvomorulin as the adhesive component of the ZA, functioned primarily in epithelia to help form the occluding barrier and to establish cell surface polarity by facilitating the assembly and localization of the ZA, and in turn the formation of the ZO (22).

The current study was undertaken to test experimentally this latter hypothesis. We asked whether the inhibition of uvomorulin function in MDCK cells would interfere selectively with the formation, stability, or function of the ZA and the ZO without affecting the establishment or stability of desmosomes, or would it interfere with all forms of cell contact.

1. Abbreviations used in this paper: Umt, an 80-kD ectoplasmic domain of uvomorulin; ZA, zonula adherens; ZO, zonula occludens.
2. The ZA has also been called the "belt desmosome", but this term is not used here so as to avoid confusion with true desmosomes, which are biochemically distinct entities.

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Materials and Methods

Purification of Canine Uvomorulin

The 80-kD ectoplasmic domain of uvomorulin (Umt) was extracted from kidney or liver tissue by a modification of the procedure of Westrheber and Kemler (37). The outer connective tissue capsules were removed from excised dog kidneys, which were then cut into several small pieces and rinsed in ice-cold PBS (0.15 M NaCl, 15 mM NaPO4, pH 7.5, 0.1 mM CaCl2, 0.1 mM MgCl2). Each kidney was then homogenized in 200 ml of ice-cold 2 mM CaCl2 without protease inhibitors in a blender (Waring Products Div., New Hartford, CT) at high speed for 1 min. The homogenate was filtered through gauze to remove remaining connective tissue and centrifuged at 7000 rpm in a rotor (model JA14; Beckman Instruments Inc., Palo Alto, CA). The pellets were resuspended to a total volume of 100 ml in 0.14 M NaCl, 1.2 mM CaCl2, 10 mM Hepes, pH 7.5, 4 mM KC1, 0.6 mM MgCl2. Trypsin and DNAse I were added from 10-x stocks in the above buffer to final concentrations of 0.2 mg/ml and 50 ng/ml, respectively, and the material was incubated shaking gently at 37°C for 1 h. The digestion was terminated by the addition of soybean trypsin inhibitor to a final concentration of 0.3 mg/ml, and large particles were removed by centrifugation at 7000 rpm for 10 min in the JA14 rotor (Beckman Instruments Inc.). All subsequent steps were carried out at 4°C. The material was then centrifuged at 30,000 rpm for 60 min in another rotor (model 70Ti; Beckman Instruments Inc.) to remove the remaining particulate material. 1 mg phenylmethylsulfonyl fluoride (PMSF) and 1 mM iodoacetamide were added to the final supernatant to inhibit nonspecific proteolysis. Liver tissue was handled similarly, except that the preparation had to be scaled up severalfold to accommodate the greater tissue mass.

The Umt was then purified from this supernatant by a batch immunoaffinity procedure. To make the immunoaffinity matrix, monoclonal antibody rrl against canine uvomorulin (21) was first purified from ascites fluid on protein A-Sepharose. It was then coupled to CNBr-Sepharose at a ratio of 2 mg/ml packed gel according to the manufacturer's instructions. Triton X-100 was added to the supernatant of the trypsin digest to a final concentration of 0.5%, which was then mixed with 1 ml packed rrl-Sepharose and incubated rotating overnight. The Sepharose beads were then pelleted at 1,000 rpm for 5 min in a table top centrifuge, washed in the original volume, and washed 3 times in 1% Triton X-100, 0.5% Na deoxycholate, 0.2% SDS, 0.15 M NaCl, 10 mM Tris, pH 7.5. They were then washed once in 0.5 M NaCl, 10 mM Tris, pH 7.5 and loaded into a small column. The Umt was eluted from the affinity matrix with 3 M NaSCN, 50 mM Tris, pH 7.5. Fractions were quickly assayed for protein content by the Amido Schwartz filter method (34). The peak fractions were pooled, dialyzed against PBS with 3 changes, and concentrated by placing the filled dialysis tubing in Acacdide II (polyethylene glycol, molecular mass = 500 kD; Calbiochem-Behring Corp., La Jolla, CA).

Production and Characterization of Anti-Uvomorulin Antibodies

One polyclonal rabbit antisem raised against canine Umt (a-umt) was used in these studies. The rabbit was immunized with ~60 µg purified Umt mixed with complete Freund's adjuvant by injection into the popliteal lymph node (18). The first boost 3 wk later was half-intradermal and half-subcutapar with ~40 µg Umt in incomplete Freund's. After another 3 wk ~40 µg was injected i.m. in PBS. 1 wk later the i.m. boost was repeated, followed 1 and 2 d later by i.v. boosts in PBS with 20 µg Umt each. The rabbit was bled 10 d after the last boost and then 3 more times at weekly intervals. Subsequent bleeds were done after a repetition of the i.m. and i.v. boosts.

The a-umt antisem was analyzed by immunoblotting. Either purified Umt or an SDS extract of MDCK cells, prepared as described (21), was separated by SDS-PAGE, electrophoretically transferred to nitrocellulose, and immunoblotted by standard procedures (6, 32). Rinsing the nitrocellulose in 20% MeOH before fixation in TCA improved the staining somewhat. The blocking step, antibody incubations, and washes were all done with Blotto buffer, containing 5% nonfat dry milk, 0.2% Triton X-100 in PBS. Alkaline phosphatase-conjugated anti-rabbit and alkaline phosphatase-conjugated anti-mouse (from Bio-Rad Laboratories, Richmond, CA) were used as secondary antibodies and the reaction was developed with the substrates 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Bio-Rad Laboratories) according to the Bio-Rad instructions.

IgG fractions were prepared from the a-umt antisem, the preimmune rabbit antisem, and monoclonal antibody rrl by purification on protein A-Sepharose using standard procedures for rabbit antisera and mouse monoclonals (7). Monovalent Fab fragments were prepared from these IgG fractions and a mouse nonimmune IgG fraction (ICN Biomedicals Inc., Costa Mesa, CA) by digestion with pepsin according to the procedure of Brackenbury et al. (5). Special care was taken to dialyze the product exhaustively to avoid toxic effects of the reagents used on treated cells. SDS-PAGE analysis of the products showed that no detectable undigested IgGs remained. The Fab fragments were dialyzed finally into 10 mM Hepes, pH 7.5, 0.14 M NaCl and stored frozen at -70°C.

MDCK Cell Growth and Resistance Measurement

Strain 1 MDCK cells were grown and passaged as stocks in MEM and 5% FCS as previously described (1, 14). For resistance measurements, cells were plated onto filter chambers as described (14), except that commercially available filter chambers were used. 2 x 105 cells were seeded onto Millicell HA (Millipore Continental Water Systems, Bedford, MA) and 1 x 105 cells were seeded onto Transwell nucleopore filters, 0.4 µm pore, 24-well plate size (Costar Data Packaging Corp., Cambridge, MA). The measurement of transepithelial electrical resistance was carried out exactly as described (14, 21), except that 4 µA was used for the stimulating pulse. In antibody treatment experiments, IgGs or Fabs were added in 1 ml of normal medium to the chambers in individual wells of a 24-well plate, and new medium (containing antibodies) was added at 24-h intervals to maintain the health of the monolayers.

Immunofluorescence Microscopy

Cells grown on either glass coverslips or on Transwell filters (Costar Data Packaging Corp.; polycarbonate based) were fixed for 4 min in -20°C MeOH, quickly rinsed in -20°C acetone, and allowed to air dry. They were then stained by indirect immunofluorescence and mounted on glass slides in a solution of Mowial (Calbiochem-Behring Corp.) by published procedures (1), except that the washes were done on an orbital shaker for agita- tion. Primary antibodies used were a mouse monoclonal to desmoplakin from Boehringer Mannheim Biochemicals (Indianapolis, IN) for desmo- somes, a rat monoclonal antibody to ZO-1 for tight junctions (35), and mouse monoclonal rrl (21) or rabbit a-umt for uvomorulin. Conjugated sec- ond antibodies were an affinity-purified Texas Red anti-mouse and an affinity-purified Texas Red anti-rabbit from Jackson ImmunoResearch Laboratories (Westbury, NY) and a FITC-conjugated rabbit anti-rat from Boehr- ringer Mannheim Biochemicals. To stain actin filaments with rhodamine-phalloidin (Molecular Probes Inc., Junction City, Oregon), a 1:10 dilution in PBS was applied to the cells for 20 min and the samples were washed like the immunofluorescence samples.

Ca2+-Switch Assay

Before use in the Ca2+-switch assay, strain 1 MDCK cells were grown in flasks for no more than 3 d to facilitate their trypsinization into single cells. After trypsinization the cells were pelleted and then washed and resus- pended in low Ca2+ medium. The low Ca2+ medium was MEM for suspension culture (S-MEM), with 2.2 g/liter NaCO3, 5 gM CaCl2 added, and 5% heat inactivated and dialyzed FCS. The low Ca2+ medium was prepared as previously described (21). Approximately 2 x 106 cells were then plated into each 35-mm culture dishes that contained four 12-mm-diam round coverslips. Cells were returned to the incubator for a period of either 5 or 24 h to allow them to attach to the coverslips.

To initiate the Ca2+-switch experiment, the low Ca2+ medium was first removed along with the remaining unattached cells. Then 1 ml of normal growth medium (MEM with 5% heat inactivated FCS) containing 200 µg/ml of either the IgG or the Fabfragments derived from either the a-umt serum or the preimmune serum was gently added to the dishes. The cells were returned to the incubator for a period of either 5 or 24 h. At the end of the incubation, coverslips were removed, rinsed quickly in PBS, fixed, and stained with the various probes.

For resistance measurements, the Ca2+-switch assay was done in a similar way, except that the cells were plated onto the Millicell HA filter chambers (Millipore Continental Water Systems). 3.5 x 105 cells were plated onto each filter, and the Ca2+-switch was initiated by scooping the chambers into a large volume of normal culture medium (MEM and FCS) in a tissue culture dish.
Results

Purification of Canine Uvomorulin and Antiserum Generation

To perform the experiments in this study, it was first necessary to generate a potent blocking rabbit antiserum to canine uvomorulin. To do so, Umt was purified from either canine kidney or liver on an immunoaffinity column of rrl-Sepharose (see Materials and Methods). Fig. 1 A shows a Coomassie-stained SDS gel of such a sample from two different preparations. A major polypeptide migrating at ~80 kD was found in each of the preparations. Immunoblotting with monoclonal antibody rrl (21) showed that this polypeptide was derived from uvomorulin (Fig. 1 B, lane a). No Coomassie-stained polypeptide bands other than Umt were visible in these preparations. Approximately 50 or 100 μg of pure Umt could be obtained by this procedure from two kidneys or one liver, respectively.

An antiserum raised against purified Umt in a rabbit by the popliteal lymph node immunization procedure had a very high titer to Umt. By ELISA assay the titer was an ~1:1,000 dilution. Immunoblot analysis showed that the antiserum recognized purified Umt (Fig. 1 B) and the entire 116-kD uvomorulin polypeptide in extracts of MDCK cells (Fig. 1 C). The lower molecular mass bands faintly stained by the antiserum in the Umt sample and the MDCK cell extract are probably minor degradation products of uvomorulin. They were still recognized by antibodies that had been microaffinity purified by the Olmstead procedure (6) on the SDS gel-purified 80-kD Umt band (Choi and Gumbiner, unpublished observations). By indirect immunofluorescence, the antiserum stained MDCK cells at the regions of cell–cell contact in a pattern indistinguishable from the staining with monoclonal antibody rrl (Fig. 1 D). By these criteria, the antiserum seems to be a high titer specific reagent for canine uvomorulin.

A Ca²⁺-Switch Assay for the Assembly of the MDCK Junctional Complex

From previous work it was evident that antibodies to uvo-
morulin could block tight junction formation and cell adhesion between MDCK cells in rapid short term assays (2, 21). Ideally, to determine the absolute dependencies of various junctions on uvomorulin-dependent adhesion mechanisms it would be valuable to know the consequences of a long term, steady-state inhibition of uvomorulin function in MDCK cells. We tried initially growing strain I MDCK cells in the presence of anti-uvomorulin antibodies for 3 d; the time required for the development of fully confluent polarized monolayers. When grown in very high concentrations of either the monoclonal antibody rrl or the polyclonal antiserum to uvomorulin (α-umt), the cells became confluent and were indistinguishable from control cultures by phase contrast microscopy (not shown). Also, by criteria of immunocytochemical staining (see below), these antibody-treated cultures formed all elements of the junctional complex (not shown). Thus, the strain I MDCK cells could eventually overcome the effects of anti-uvomorulin antibodies. This finding therefore precluded using long-term growth in culture as an assay for these studies.

We therefore used the Ca²⁺-switch assay developed by Gonzalez-Mariscal to investigate the de novo formation of tight junctions in strain II MDCK cells (17). Similar assays have also been used to study the de novo assembly of desmo-
somes and the ZA in MDCK cells and in other cell types (19, 31). The formation of each of the different elements of the junctional complex in MDCK cells grown on coverslips during a Ca\textsuperscript{2+}-switch experiment was evaluated by immunofluorescence staining of a component specific to each junction (Fig. 2). A monoclonal antibody to ZO-1, a cytoplasmic peripheral membrane protein associated with the ZO (35), was used as a marker to follow the formation of the tight junction (Fig. 2, A–C). In cells maintained in low Ca\textsuperscript{2+}-medium, ZO-1 staining was barely detectable over background and appeared diffusely distributed in the cell (Fig. 2 A). 5 h after return to normal Ca\textsuperscript{2+}-containing medium (MEM), ZO-1 was found localized to a very sharp continuous band around the cell periphery at the regions of cell–cell contact (Fig. 2 B). After 24 h the pattern of ZO-1 was unchanged except that the staining appeared more intense. This pattern was typical of the distribution of ZO-1 in confluent monolayers of MDCK cells (35).

The accumulation of desmoplakin, a protein of the cytoplasmic plaque of all desmosomal junctions, at the plasma membrane at regions of cell–cell contact has been shown to be a good indicator of desmosome formation in a variety of cell types (8, 19, 31). Therefore, staining with a monoclonal antibody to desmoplakin was used to visualize desmosome formation in the Ca\textsuperscript{2+}-switch assay (Fig. 2 D–F). As observed for other cells, desmoplakin staining in MDCK cells kept in low Ca\textsuperscript{2+} medium was diffuse in the cytoplasm with some bright patches clustered toward the center of the cell (Fig. 2 D). Within 5 h after the return of Ca\textsuperscript{2+}, desmoplakin appeared in belts at the sites of cell–cell contact and completely circumscribed the cells (Fig. 2 E). Discrete punctate dots of staining that are thought to represent individual desmosomes were also observed. The distribution after 24 h in normal medium was similar (Fig. 2 F), and like ZO-1, the staining was a little brighter.

Fluorescent staining of the peripheral circumferential bundle of actin filaments was used as a marker for the ZA (19, 29, 39). In strain II MDCK cells, the peripheral belt of actin filaments has been shown to be disrupted by the opening of cell junctions induced by the removal of extracellular Ca\textsuperscript{2+} (29). We also found a strong peripheral ring of actin filaments in confluent strain I MDCK cells by staining with rhodamine-phalloidin (Fig. 2 I). In cells kept in low Ca\textsuperscript{2+} medium phalloidin staining of actin filaments was bright but diffusely distributed (Fig. 2 G). After 5 h in Ca\textsuperscript{2+}, a thin but continuous band of actin filaments appeared at the cell periphery at the sites of cell–cell contact (Fig. 2 H). At 24 h after the Ca\textsuperscript{2+}-switch the peripheral actin belt was much broader and more intensely stained (Fig. 2 I).

The distribution of uvomorulin was also examined during the time course of junction assembly in the Ca\textsuperscript{2+}-switch experiment. Although uvomorulin is an integral membrane glycoprotein, the pattern of its accumulation at the regions of cell–cell contact was similar to the junctional markers. As observed previously (22), the distribution of uvomorulin was diffuse in low Ca\textsuperscript{2+} medium, and some clustering toward the center of the cell was evident (Fig. 2 J). Because the cells were permeabilized during fixation in this experiment, it is not possible to tell whether some of the uvomorulin molecules in low Ca\textsuperscript{2+} were present in intracellular vesicles. After 5 h in Ca\textsuperscript{2+}-containing medium, most of the uvomorulin molecules redistributed to the regions of cell–cell contact (Fig. 2 K). The pattern after 24 h was similar, with a somewhat greater extent of redistribution to the periphery (Fig. 2 L).

The redistributions of all of these markers were very rapid and dramatic and occurred in the vast majority of cells in the culture. Initial steps in their formation began 15–30 min after the addition of Ca\textsuperscript{2+}, and the staining patterns observed at 5 h occurred in most of the monolayer within 2 h (Ogas, Swedlow, Agard, and Gumbiner, unpublished observations). The 5 and 24 h time points shown here were taken as the best ones for evaluating the effects of antibody treatments.

**Antibodies to Uvomorulin Inhibit the Formation of All Junctions Cooperatively**

The Ca\textsuperscript{2+}-switch assay was used to determine the effect of anti-uvomorulin antibodies on the formation of specific junctional elements. Polyclonal antibodies to canine uvomorulin (α-umt) were used because they did not interfere with subsequent indirect immunofluorescent staining with the mouse monoclonal antibody to desmoplakin or the rat monoclonal to ZO-1. Either purified IgGs (Fig. 3) or Fab fragments (Fig. 4) were added to the cells at the same time as the Ca\textsuperscript{2+}-containing medium. At either 5 or 24 h after antibody and Ca\textsuperscript{2+} addition, the cells were fixed and stained with antibodies to desmoplakin or ZO-1, or with rhodamine-phalloidin to observe the extent of formation of the junctional elements. Preimmune IgG or preimmune Fab fragments had no discernable effect on the extent of junction formation. In fact, the data in Fig. 2 were from the preimmune controls.

When cells were treated with α-umt IgG for 5 h, there was significant, but partial inhibition of the formation of all junctional elements (Fig. 3, A, C, and E). The recruitment of ZO-1, desmoplakin, and actin filaments to the cell periphery were all inhibited to a similar extent relative to preimmune controls (Fig. 2). In contrast, after 24 h the cells were able to overcome the effects of α-umt IgG treatment, and their ability to form all of the junctions recovered (Fig. 3, B, D, and F). The redistributions of ZO-1, desmoplakin, and actin filaments to their peripheral location was similar to controls (Fig. 2). The reasons why junction formation recovered at long times in the presence of the IgG is not known (see Discussion). These results do demonstrate, however, that the initial inhibition by and the subsequent recovery from α-umt treatment was not selective for any of the three junctional elements. All behaved cooperatively.

Fab fragments derived from α-umt were more potent inhibitors of junction formation than the IgGs (Fig. 4). 5 h of α-umt Fab treatment almost completely inhibited the formation of all three junctional elements (Fig. 4, A, C, and E). Recruitment of desmoplakin, ZO-1, and actin filaments to the cell periphery only occurred in small patches and only in a small fraction of the cells. The redistribution of the markers to the periphery occurred to a greater extent after 24 h, but was still only partial compared to controls (Fig. 4, B, D, and F). The inhibition at 24 h was similar to the 5-h time point with α-umt IgG (Fig. 3, A, C, and E). Again, the extent of inhibition and recovery of the formation of the three junctions was coordinate. No selective action of the Fab fragments on any of the markers was notable.

Recovery of junction formation after periods of antibody treatment could occur even in the absence of a functional

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Figure 3. Inhibition of junction formation by α-umt IgG. The Ca\(^{2+}\)-switch experiment was done as in Fig. 2 except that 200 μg/ml α-umt IgG was included in the Ca\(^{2+}\)-containing medium added to the cells to initiate junction assembly. (A, C, and E) 5 h after switch to Ca\(^{2+}\)-containing medium. (B, D, and F) 24 h after switch to Ca\(^{2+}\)-containing medium. Cells were stained with (A and B) mAb to ZO-1, (C and D) mAb to desmoplakin, and (E and F) rhodamine-phalloidin. Bar, 20 μm.

uvomorulin molecule, if alternate adhesion mechanisms exist in MDCK cells. Although there is no direct molecular test to discriminate between functional and nonfunctional uvomorulin molecules, the accumulation of uvomorulin at regions of cell–cell contact seems to be indicative of its participation in an adhesive interaction (9, 22, 23). Therefore its distribution in antibody treated cells was examined by immunofluorescence microscopy. In such an experiment a fraction of the uvomorulin may already be bound by the antibodies used in the experimental treatment. Therefore the distributions of both the treating antibody (Fig. 5, A, C, E, and G) and the epitopes accessible to staining with monoclonal
Figure 4. Inhibition of junction formation by α-umt Fab fragments. The Ca²⁺-switch experiment was done as in Fig. 2 except that 200 μg/ml α-umt Fab fragments were included in the Ca²⁺-containing medium added to the cells to initiate junction assembly. (A, C, and E) 5 h after switch to Ca²⁺-containing medium. (B, D, and F) 24 h after switch to Ca²⁺-containing medium. Cells were stained with (A and B) mAb to ZO-1, (C and D) mAb to desmoplakin, and (E and F) rhodamine-phalloidin. Bar, 20 μm.

rrl (Fig. 5, B, D, F, and H) were examined. The treating antibody was not found localized to the cell periphery at either 5 or 24 h, whether it was IgG (Fig. 5, E and G) or Fab (Fig. 5, A and C). The distribution of Fab fragments appeared diffuse (Fig. 5, A and C), but the IgG staining was in scattered bright punctate dots (Fig. 5, E and G). Cells treated with preimmune antibodies were only faintly labeled and did not show either pattern of staining (not shown). These patterns suggest that the Fab fragments were bound to uvomorulin molecules uniformly distributed over the cell surface away from the regions of cell–cell contact, and that the IgGs were endocytosed after binding to uvomorulin and accumu-
Figure 5. Distributions of uvomorulin and α-umt in the antibody-treated cells. Samples from the experiments shown in Figs. 4 and 5 were stained either directly with fluorescent anti-rabbit IgG second antibodies to visualize the treating α-umt antibodies or Fab fragments (A, C, E, and G) or with mAb r11 to uvomorulin and a fluorescent anti-mouse IgG second antibody (B, D, F, and H). (A and B) Cells treated 5 h with α-umt Fab fragments. (C and D) Cells treated 24 h with α-umt Fab fragments. (E and F) Cells treated 5 h with α-umt IgG. (G and H) Cells treated 24 h with α-umt IgG.
Treatment of Tight, Confluent Monolayers of MDCK Cells with Blocking Antibodies to Uvomorulin

Several investigators have reported that antibodies to uvomorulin, or its homologue, could completely dissociate epithelial cells grown in culture (2, 3, 9, 30, 40). Antibody rrl was found to have such activity on MDCK cells (Gumbiner, Behrens, and Vestweber, unpublished observations). However, such experiments have usually been performed on subconfluent or barely confluent cells. Also, no attempts were made to assess the extent to which junctional complexes had been formed by the cells before antibody treatment. We wished to determine whether MDCK cells with well-formed junctional complexes were susceptible to dissociation by antibodies to uvomorulin.

Strain I MDCK cells were grown for 4 d on filters to test their susceptibility to dissociation by anti-uvomorulin antibodies. The transepithelial electrical resistance was used as the most sensitive measure of cell dissociation. Even the introduction of submicroscopic gaps between the cells, which are large enough to permit the passage of small ions, will increase the electrical conductance of the monolayer (26, 27). Also, because of the electrical nature of the measurement, very small numbers of such gaps (or larger ones) will cause very large drops in resistance (13, 26). Fig. 6 D shows a graphical representation of the relationship between the loss of electrical resistance and the fraction of the cells in the monolayer with electrically permeable junctions, calculated from the equation (13):

$$\frac{1}{R_t} = f_o/R_o + f_c/R_c$$

where $R_t$ = the overall resistance of the monolayer, $R_o$ = the resistance of open regions of the monolayer, $R_c$ = the resistance of closed regions of the monolayer, $f_o$ = the fraction of cells in the monolayer with opened junctions, and $f_c$ = $1 - f_o$ = the fraction of cells in the monolayer with closed junctions. This relationship gives an estimate of the maximum fraction of the cells that could be considered dissociated, with the remainder of the cells having optimally sealed tight junctions. It is also possible that antibody treatment could cause a more uniform increase in the leakiness of the tight junctions in all cells in the monolayer. Such uniform changes, albeit interesting, would not be an indication of physical dissociation of the cells.

mAb rrl, α-umt IgG, preimmune IgG, or Fab fragments derived from all of these antibodies were added in high concentrations to both sides of the monolayers (Fig. 6, A-C). When added to the cells for a period of 1 d, none of these antibodies caused a decrease in transepithelial electrical resistance that would be expected for any substantial extent of cell dissociation (Fig. 6 A). Because the polyclonal α-umt antibodies did cause a small decrease in resistance, they were added to the monolayers for a longer time. When incubated for 2 d with a fresh replacement of antibodies and medium after the first day, no significant decrease in resistance was observed (Fig. 6 B). Several experiments were also carried out to test whether the effects of antibodies would be transient and therefore of shorter duration. At 6 h of antibody treatment, only the IgG fraction of α-umt caused a significant decrease in the transepithelial electrical resistance. Fig. 6 C shows the most dramatic effect that was observed in several
repetitions of the same experiment. Using the equation above and taking $R_c = 2,000 \text{ ohm} \times \text{cm}^2$ for this experiment, it can be calculated that a maximum of 13% of the cells in this monolayer were separated sufficiently by $\alpha$-umt IgG to cause their junctional complexes to become electrically leaky. Alternatively, this antibody may have increased the junctional permeability in most of cells of the monolayer without causing any cell dissociation. Further experimentation using a good quantitative assay for physical separation in addition to the electrophysiological measurements would be required to distinguish between these two alternatives. Therefore, the IgG fraction of the polyclonal $\alpha$-umt antiserum caused at most a very transient dissociation of 13% of the MDCK cells in tight, confluent monolayer.

The lack of effect of mAb rrl and the $\alpha$-umt Fab fragments on these monolayers cannot be attributed to a problem with their penetration through the filter or the basolateral intercellular spaces. Fig. 7 shows that a significant amount of the experimental antibodies did penetrate into the intercellular spaces and bind to uvomorulin on the cell surface. Therefore, these active blocking antibodies to uvomorulin could bind to uvomorulin on the lateral surface of MDCK cells without causing extensive cell dissociation or long lasting perturbation of the permeability of the ZO.

Discussion

Experiments were carried out to determine whether the inhibitory action of anti-uvomorulin antibodies was selective for a subset of junctional elements. Using a Ca$^{2+}$-switch assay with cultured MDCK cells and specific immunocytochemical markers for the ZO, the ZA-associated actin filament bundle, and the desmosomes, we could not find conditions of inhibition with antibodies to uvomorulin under which the formation of the different junctions was separable. Because the immunofluorescence observations used in these experiments were not quantitative in nature, it would not have been possible to detect small differences in the rate of formation or the extent of inhibition of the junctions. However, the qualitative conclusion derived from these experiments is that the formation of all three junctional elements are similarly dependent on the function of uvomorulin at the cell surface. The adhesive function of uvomorulin seems to be required for the formation of all intercellular contacts. These findings do not support a parallel model for junction assembly between epithelial cells as shown in Fig. 8 B. They are more consistent with a hierarchical model for the formation of intercellular contacts between epithelial cells (Fig. 8 A).

An important caveat to the support of the hierarchical model is the possibility that specific inhibition of the adhesive functions of other junctional elements could, in theory, have the same effects as blocking uvomorulin function. We have not been able to do the reciprocal experiment to test whether the inhibition of desmosome assembly with blocking antibodies to desmosomal surface glycoproteins, which are presumably adhesive molecules, would slow or block the formation of the ZA and/or the ZO. Unfortunately, very few experiments on the inhibition of desmosome formation by antibodies have been reported (8, 25), and blocking antibodies to the desmosomal glycoproteins are not widely available. Until such experiments are done it remains possible that all the junctional elements are interdependent during the development of intercellular adhesion and none has precedence over the other. The findings presented here do rule out, however, that the function of uvomorulin is selective for the assembly of the ZA and the ZO.

To determine the absolute dependencies of the formation of different junctions on uvomorulin-dependent adhesion, it would be valuable to examine the effects of a long-term, steady-state inhibition of uvomorulin function. Because we found that the inhibitory activity of antibodies was eventually overcome with time, we used a kinetic assay for de novo junction assembly. This approach to the problem is not uncommon, since most adhesion assays employing blocking antibodies have been kinetic assays (5, 11, 40). Our observations that all three junctions were inhibited to the same extent under varying conditions and times of antibody treatment strengthens the validity of conclusions drawn from the use of a kinetic assay.

We do not know the reasons why MDCK cells overcame the effects of the blocking antibodies. The ability of cells to overcome the effects of blocking antibodies to uvomorulin, however, is not unique to the MDCK cell line or to our antibodies. Similar results have been found in other systems. The inhibition of compaction of early mouse embryos by anti-uvomorulin was eventually overcome with time despite the continual presence of antibody (24, 37). Also, antibodies to uvomorulin, which blocked compaction of mouse embryos and adhesion between cultured cells, failed to prevent cell
junctional complex. In this model all of the junctions between epithelial cells participate in the overall adhesion and recognition between epithelial cells, and adhesion molecules are the same as intercellular adhesive proteins of junctions before their localization into morphologically discernible structures. In this version, the ZA is proposed to facilitate the formation and localization of the ZO independent of the formation of desmosomes.

adhesion, epithelialization, and the normal morphogenesis of kidney tubules in vitro (38). Despite the fact that antibodies to L-CAM altered the patterning of dermal condensations in feather germs undergoing induction in the chick embryo, they did not directly affect dermal cell adhesion (15). Thus functional recovery from an antibody block seems to be typical of uvomorulin-mediated adhesion.

Many investigators have shown that specific perturbation of uvomorulin with antibodies causes cells in culture to dissociate (2, 3, 9, 30, 40). These findings could have important implications for the regulation of the epithelial junctional complex in vivo. They suggest that cellular regulation of uvomorulin function alone would be sufficient to control the state of the entire junctional complex. However, experiments have generally failed to demonstrate the dissociation of cells from intact, differentiated epithelial tissues with antibodies to uvomorulin or L-CAM (15, 38). Perhaps once the cells become firmly held together by other junctions, uvomorulin has a less important role in maintaining intercellular adhesion.

We re-examined this problem with the MDCK cultured cell line under a state that approached a more differentiated epithelial tissue. When grown under optimal conditions for the establishment of a highly polarized epithelial cell monolayer with well-formed junctional complexes, the strain I MDCK cells were very refractory to dissociation by various antibodies to uvomorulin. Using a very sensitive electrical measurement we could not detect extensive dissociation of confluent MDCK cells grown on filters with several different antibodies and Fab fragments that we know block junction formation. Even if the decrease in resistance caused by treatment with α-umt IgG at 6 h was due to cell dissociation, it was only transient and occurred in at most 13% of the cells. Our difficulty in causing extensive cell dissociation in comparison to previous reports is probably not attributable to differences in the anti-uvomorulin antibodies used. Our antibodies were of very high titer and showed potent blocking activities in other assays, including a resistance recovery assay (21), the Ca²⁺-switch assay in the present work, and in

4. One possible exception in an analogous but not identical system is the dramatic dissociation of somites excised from chick embryos by antibodies to the related neural Ca²⁺-dependent adhesion molecule, N-cadherin (10). the dissociation of subconfluent MDCK cells (Gumbiner, Vestweber, and Behrens, unpublished observations). Rather, the conditions of cell growth into a well-formed model epithelium is probably responsible for the different results.

Behrens et al. reported that strain II MDCK cells grown on filters could be dissociated by arc-1, a monoclonal antibody to canine uvomorulin (2). Their findings are not directly comparable to ours, however, because the cells were grown only overnight, just to confluence, and a quantitative assay to estimate the extent of the effect was not used. Interestingly, they reported that dissociability was prevented by treating the cells with colchicine or drugs that raise the levels of intracellular cAMP. This suggests that dissociability by antibodies to uvomorulin is influenced by the health or metabolic state of the cells. It is possible, therefore, that cells in demanding metabolic conditions, such as rapidly growing tumor cells or cells undergoing developmental alterations, may be more dependent on an uvomorulin-dependent adhesion mechanism.

Although we found that several antibody reagents could bind to uvomorulin at the lateral cell surfaces without causing a decrease in transepithelial resistance, it is still possible that uvomorulin does normally control the state of the junctional complex in differentiated epithelia. Under these conditions uvomorulin could exist in a form of adhesive contact (perhaps the ZA) that is not easily modulated by the binding of an antibody. Also, the nature of antibody binding to uvomorulin on the cell surface might influence the cellular response. For example, only the polyclonal α-umt IgGs caused a transient loss in transepithelial resistance. This seems surprising, since the IgGs were less effective than the Fab fragments in the Ca²⁺-switch assay and also have the potential to cross-link cells together. Perhaps the bivalent polyclonal antibody induced uvomorulin internalization (consistent with the data in Fig. 5, E and G), and thereby modulates its function. The transient loss of transepithelial resistance caused by the α-umt IgG either could be due to the physical dissociation of a small proportion of the cells, or could indicate that uvomorulin regulates in a more subtle way the state of the junctional complex. Alternatively, cross-linking a cell surface glycoprotein like uvomorulin could...
perturb tight junctions indirectly and nonspecifically by altering the cortical membrane cytoskeleton.

How might the finding that the assembly of all members of the junctional complex depends on the functions of uvomorulin be interpreted? Four major explanations, though not mutually exclusive, are offered here. The first is that the adhesive strength of uvomorulin is required to maintain intercellular contacts and that the adhesiveness of desmosomes and tight junctions is minimal and insufficient to hold cells together. Second, in addition to adhesion, uvomorulin might have an important role in intercellular recognition (11, 15, 36). It could transmit a signal in response to contact formation that instructs the cell to turn on the normal program of junctional development. Third, perhaps only the ZA (using uvomorulin as its primary adhesive component) and its associated actomyosin filament system can exert the initial force that can bring cells into close enough contact to allow the assembly of the other junctions. This hypothesis predicts that desmosomes and tight junctions might form in the absence of uvomorulin function if the cells could be brought artificially into close enough contact. Finally, there could be a direct interaction between the cytoskeletal elements involved in the assembly of the different junctions. Support for this hypothesis comes from the observations that actin filaments and intermediate filaments seem to be associated during the process of desmosome formation by keratinocytes (19). To distinguish between these alternatives, further experimentation on the functional relationships between the various junctional elements and adhesion molecules will be required.

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