A Functional Assay for Proteins Involved in Establishing an Epithelial Occluding Barrier: Identification of a Uvomorulin-like Polypeptide

Barry Gumbiner and Kai Simons
European Molecular Biology Laboratory, 6900 Heidelberg, Federal Republic of Germany. Dr. Gumbiner's present address is Department of Pharmacology, University of California, San Francisco, San Francisco, California 94143.

Abstract. A functional assay has been developed to identify cell surface proteins involved in the formation of epithelial tight junctions. Transepithelial electrical resistance was used to measure the presence of intact tight junctions in monolayers of Madin-Darby canine kidney (MDCK) cells cultured on nitrocellulose filters. The strain I MDCK cells used have a transmonolayer resistance >2,000 ohm cm². When the monolayers were incubated at 37°C without Ca²⁺, the intercellular junctions opened and the transmonolayer resistance dropped to the value of a bare filter, i.e., <40 ohm cm². When Ca²⁺ was restored, the cell junctions resealed and the resistance recovered rapidly. Polyclonal antibodies raised against intact MDCK cells inhibited the Ca²⁺-dependent recovery of electrical resistance when applied to monolayers that had been opened by Ca²⁺ removal. Cross-linking of cell surface molecules was not required because monovalent Fab’ fragments also inhibited. In contrast, a variety of other antibodies that recognize specific proteins on the MDCK cell surface failed to inhibit the recovery of resistance. Monoclonal antibodies have been raised and screened for their ability to inhibit resistance recovery. One such monoclonal antibody has been obtained that stained the lateral surface of MDCK cells. This antibody, r11, recognized a 118-kD polypeptide in MDCK cell extracts and an 81-kD fragment released from the cell surface by trypsinization in the presence of Ca²⁺. Sequential immunoprecipitation with antibody r11 and a monoclonal antibody to uvomorulin showed that this polypeptide is related to uvomorulin. The role of uvomorulin-like and liver cell adhesion molecule (L-CAM)-like polypeptides in the establishment of the epithelial occluding barrier is discussed.

Many transporting epithelia generate and maintain large ionic and molecular compositional differences between the luminal and serosal spaces of tissues. Vectorial transport of ions and molecules across the epithelium arises from the polarized distribution of channels and transport proteins in the epithelial cell surface. Plasma membrane proteins are segregated into two distinct domains; the apical domain facing the lumen and the basolateral domain facing the serosal compartment (Simons and Fuller, 1985). To maintain compositional differences between the luminal and serosal spaces, the epithelium as a whole also behaves as a selective permeability barrier.

The epithelial permeability barrier results not only from the properties of the epithelial cells, but also from the formation of intercellular junctions that inhibit diffusion across the epithelium through the paracellular spaces. The epithelial junctional complex consists of three morphologically distinct elements, the zonula occludens (tight junction), zonula adherens (intermediate junction), and desmosomes (Farquhar and Palade, 1963). Cell adhesion molecules of epithelial origin such as liver cell adhesion molecule (L-CAM)1 or uvomorulin can also be considered part of this complex (Edelman, 1984).

The zonula occludens or tight junction, is believed to be primarily responsible for the paracellular permeability barrier (Claude, 1978; Diamond, 1977; Farquhar and Palade, 1963; Martinez-Palomo et al., 1980). The tight junction forms at the border between apical and basolateral membranes (see review by Simons and Fuller, 1985), and has been proposed to form a localized barrier in the membrane that prevents the intermixing of membrane proteins between domains (Diamond, 1977; Meldolesi et al., 1978; Pisam and Ripoche, 1976). It is not yet known to what extent the other elements of the epithelial junctional complex participate in the formation of an effective occluding barrier. Nor is it understood how they participate in the polarization of the epithelial cell surface.

To identify specific protein molecules involved in the formation of an effective epithelial occluding barrier, we have developed a functional assay based on the studies of Martinez-Palomo et al. (1980). It employs a very sensitive measure of the integrity of the paracellular occluding barrier, the transepithelial electrical resistance. The Madin-Darby canine kidney (MDCK) strain I cells used in the assay exhibit a transmonolayer resistance of 2,000–10,000 ohm cm². These values

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457
of transepithelial resistance depend on the presence of well-developed tight junctions (Claude, 1978; Diamond, 1977). The resistance of the monolayer therefore can be used to assay the formation of the tight junctions as the endpoint in the establishment of a functional occluding barrier, although other junctional elements may also participate in the assay.

It is well known that epithelial junctions can be disrupted by the removal of calcium ions from the bathing medium (Kartenbeck et al., 1982; Meldolesi et al., 1978; Pietlik et al., 1983; Sedar and Forte, 1964). Martinez-Palomo et al. (1980) have shown that the tight junctions of low-resistance MDCK strain II cells can be reversibly opened by manipulating extra-cellular Ca\(^{2+}\) concentration. Using optimal conditions for the reversible disruption of the tight junctions of high resistance strain I MDCK cells, we have looked for antibodies that block the reformation of tight junctions. Here we describe the assay in some detail and provide evidence for its specificity. The first monoclonal antibody found to be functional in this assay is presented along with the preliminary characterization of the molecule it recognizes in MDCK cells.

**Materials and Methods**

**Chemicals**

Trypsin (trypsin-TPCK) and soybean trypsin inhibitor were purchased from Worthington Biochemical Corp. (Freehold, NJ). Phenylmethylsulfonfyl fluoride, iodoacetamide, Hepes, EDTA, and EGTA were obtained from Sigma Chemical Co. (St. Louis, MO). SDS was from Serva (Heidelberg) and Triton X-100 was from Merck (Darmstadt, FRG). Cell culture media and reagents were from Gibco (Glasgow).

**Growth of Strain I Cells on Filters**

Strain I MDCK cells, subclone 8-1B, were maintained as stocks in Eagle's minimal essential medium with Earle's salts (MEM) with 5% fetal calf serum and 10 mM Hepes in plastic Falcon flasks (Falcon Labware, Oxnard, CA) and passed routinely as before (Balcarova-Stander et al., 1984; Fuller et al., 1984). The conditions for growth on nitrocellulose filters in mini-Marbrook chambers (Hendley Engineering, London) have been described in detail (Fuller et al., 1984). In the present experiments Millipore 0.45-μm HAFT filters (Millipore Corp., Molsheim, France) were always used. 8-10 filter monolayers (~3 cm\(^2\) each) were prepared from each confluent 75-cm\(^2\) flask of cells. They were grown for at least 4 d before use in experiments. Sets of filters were maintained sometimes in culture for 5 or 6 d before being used; when this was done, half of the growth medium (~35 ml) was replaced on day 4 with serum-free growth medium. Sometimes in culture for 5 or 6 d before being used; when this was done, half of the growth medium (~35 ml) was replaced on day 4 with serum-free growth medium.

Strain I MDCK cells were also grown in 96-well Millititer HA filtration plates (Millipore Corp.). The 96-well plates have been designed primarily for the filtration of multiple samples. The usual product is manufactured with a sheet of porous hydrophobic material attached underneath to the nitrocellulose filter. Although cells could be grown in the plates when this sheet was present, the sheet interfered with the washing of ions from the monolayer that was required for the resistance recovery assay described in this paper. The best solution to this problem was to obtain by special order from the Millipore Corp. the same sterile Millititer-HA plates without the hydrophobic sheet (catalogue No. SA21135E4). To provide adequate access of culture tissue medium to the filter and hence the cell monolayer, it was necessary to modify the filter plate itself. Using sterile hemostat clamps, chunks of plastic were broken out from the edges of the plate. The four corners were left intact to support the plate. Filter plates were maintained in culture individually. Each plate was placed into a sterile 245 × 245 × 20 mm bioassy dish (Nunc, Roskilde, Denmark). 200 ml growth medium was added to the dish outside the plate. 1.5 confluent 75 cm\(^2\) flasks of cells were passed into each 96-well plate. Each flask of trypsinized cells was resuspended into 13 ml culture medium and 0.2 ml was put into each of the 96 wells (0.38 cm\(^2\) each). Cells grown on filter plates were always maintained for at least 4 d before use. If plates were maintained longer in culture, i.e., up to 7 d, 75 ml of medium was replaced with fresh serum-free medium on day 4.

**Resistance Recovery Assay, Mini-Marbrook Chambers**

Transmonolayer electrical resistance across MDCK monolayers grown in mini-Marbrook chambers was measured with a device similar to that described by Mazur and Handler (1981). In brief, current, which was measured across the monolayer through two pairs of saturated KCl-3% agar bridges. Current was passed between two silver-silver chloride electrodes and voltage was measured with two calomel electrodes. Resistance was calculated from the change in voltage across the monolayer induced by a short (~2 s) 20-μA pulse of current. The resistance was multiplied by the surface area of the filter to yield the areal resistance in ohms square centimeters. Resistance measurements were always done at room temperature in phosphate-buffered saline, Dulbecco's formulation (PBS), either with (PBS+) or without (PBS-) 1.2 mM CaCl\(_2\) and 0.8 mM MgCl\(_2\).

The Ca\(^{2+}\)-free medium used to open the intercellular junctions consisted of minimal essential medium with spinner s (SMMEM, Gibco), 0.2 g/liter Na\(_2\)CO\(_3\), 10 mM Hepes, 1% dialyzed fetal calf serum, and was brought to pH 7.4 with NaOH. Fetal calf serum was heat inactivated at 56°C for 30 min and dialyzed in the cold against 0.15 M NaCl, then 0.15 M NaCl with 0.2 mM EDTA, and then two changes with 0.15 M NaCl. Each change was against a 100-fold volume for 12 to 24 h. The Ca\(^{2+}\)-free medium was always stored in plastic containers. The Ca\(^{2+}\)-containing recovery medium was made by slowly adding 1 ml 0.1 M CaCl\(_2\) to each 100 ml Ca\(^{2+}\)-free medium while stirring.

Before the intercellular junctions were opened by the Ca\(^{2+}\)-free incubation, the resistance of each monolayer was measured. The filters were rinsed quickly in room temperature PBS+ and the resistance was measured in PBS+. Only monolayers with resistances >2,500 ohm·cm\(^2\) were used for experiments. To open the intercellular junctions after the resistance was measured, the filters were rinsed quickly in two beakers of PBS- and then immersed at time 0 min into 30-40 ml of Ca\(^{2+}\)-free medium in small glass beakers kept at 37°C in a water bath. During all incubations at 37°C in both Ca\(^{2+}\)-free medium and in recovery medium, the filter chambers were allowed to float to the top of the incubation medium. This avoided the development of large pressure differences between the two sides of the monolayers. After a timed incubation, the transmonolayer resistance was measured in PBS+. Depending on the experiment, the filters were then either returned to the Ca\(^{2+}\)-free medium for longer incubation, immersed in Ca\(^{2+}\)-containing medium at 37°C for a recovery incubation, or subjected to an intermediate experimental treatment.

To test the effects of trypsin or antibodies on the recovery of transmonolayer resistance, a 20-min intermediate treatment period was included between the Ca\(^{2+}\)-free incubation to open intercellular junctions and the recovery incubation in the presence of Ca\(^{2+}\). Ca\(^{2+}\)-free incubation was for 15 to 18 min depending on the batch of filters (see Results). After the resistance was measured the filters were gently shaken to remove excess solution and then were placed apical side up at room temperature into six-well cell culture plates (Nunc). The experimental solutions containing trypsin or antibodies in 200 μl were pipetted into the mini-Marbrook chambers, i.e., added to the apical side. The mini-Marbrook chambers were covered with moist pieces of filter paper (3-MM disks, Whatman Inc., Maidstone, England) to keep the filters humidified. The filters were rocked gently back and forth for 20 min at room temperature. Antisera were always heat inactivated at 56°C for 30 min before use. Antibody dilutions from serum, ascites fluid, or IgG solutions in PBS were always made up in the recovery medium containing Ca\(^{2+}\). The dilutions were kept at 4°C until use. Trypsin was made to 200 μg/ml in PBS- and kept at room temperature before use. After the treatment the filters were replaced in PBS- containing soybean tryptase inhibitor (after the trypsin treatment) and immersed in 37°C Ca\(^{2+}\)-containing recovery medium. The resistance of each filter was measured in PBS- after 15 min recovery incubation and at intervals of ≥15 min thereafter. 12-15 filters could be handled at a time in this assay. Usually, four or five samples in triplicates were assayed together. Accurate timing of the incubations was best accomplished by manipulating the filters sequentially at 1-min intervals at every stage of the experiment.

**Resistance Recovery Assay, Millititer Plates**

The transmonolayer resistance of cells grown in the wells of the Millititer plate was measured with a simple modification of the device used for the mini-Marbrook chambers. Resistance was measured across each of the 96 wells with the same sets of Agar-KCl bridges and electrodes, but with a different chamber. The assay chamber was a shallow plastic rectangular dish slightly larger than the Millititer plate with pegs in the four corners to hold the Millititer plate up against a lip of the well. To bathe the bottom surface, PBS was added to the chamber after the Millititer plate had been mounted. Two of the agar-KCl bridges, one connected to the current passing electrode and one from the voltage-measuring electrode, were slid underneath the Millititer plate through small holes in the
The effects of antisera and hybridoma supernatants on the recovery of resistance were assayed using the same protocol as described for the standard assay using the mini-Marbrook chambers except that the means of washing and incubation were changed. All rinses and incubations were done in 800–900 ml contained within 1-liter glass beakers. The plates were immersed vertically and slowly, taking care to avoid trapping air bubbles in the wells. A glass rod or Pasteur pipette was then agitated vigorously in front of the wells of the plate for several seconds to wash the solution out of the wells. Before antibodies were added after the 37°C Ca²⁺-free incubation, medium was removed quickly from the wells with a 12-toothed aspirator. 30–40-μl test samples were then transferred from a standard 96-well cell culture plate to the corresponding wells of the Millipore plate using a 12-channel micropipette (Tittertek, Elfab Oy, Finland). The plates were covered with their own lids and incubated, rocking, at room temperature for 20 min.

The transmonolayer resistances of all 96 wells were measured at a single point 30 to 45 min after recovery at 37°C in Ca²⁺-containing medium. The values changed very little during the measuring period because the measurement was done in <10 min at room temperature. The active polyclonal anti-MDCK antisera and an inactive preimmune serum were included in every test plate as a control. Hybridoma supernatants were supplemented with 10 mM Hepes and assayed directly. Because all samples were tested in triplicate, 30 hybridoma supernatants and the 2 controls could be assayed at one time.

**Antibody Production**

Strain 1 MDCK cells always served as the immunogen. Cells for immunization were passaged at higher than normal density (split ratio 1 to 3) and grown for 2 d. In this way, almost-confluent monolayers were obtained that could be detached from the dish without trypsin. Cells were rinsed twice and harvested with 2.0 mM EDTA in PBS at 37°C. As soon as the cells became detached from the plastic (~10–15 min), they were pelleted and resuspended in a small volume of PBS at 4°C.

Rabbits were immunized with 5 × 10⁶ cells in complete Freund's adjuvant by injection into the popliteal lymph node (Goudie et al., 1966). They were boosted every 2 wk thereafter with the same number of cells in incomplete Freund's adjuvant once intradermally and thereafter with subcutaneous injections. The cultures were prepared from bleeds taken after every boost, heat-inactivated at 56°C for 30 min, and tested in the resistance assay. Sera taken after the third boost were found to be active in the assay and therefore were used primarily in these experiments. IgG was purified from the immune and preimmune rabbit serum by affinity purification on protein A-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) using the recommended procedure. Fab' fragments were produced by papain digestion (Mage, 1980). The undigested IgG was purified away on protein A-Sepharose. SDS PAGE showed no residual IgG in the Fab' fractions.

For monoclonal antibody production, BALB/c mice were immunized intraperitoneally with 2 × 10⁷ intact cells in PBS. The mice were boosted with the same dose after 3 wk. 1 wk later, serum samples were taken, heat inactivated at 56°C for 30 min, and tested for activity in the resistance recovery assay. The two most positive mice were again boosted 4 wk after the previous boost. Their spleens were removed 3 d later and fused with the Ag8 myeloma cell line. Hybridoma production, selection, and growth were done by standard procedures (Campbell, 1984). The positive hybridoma was subcloned twice by limiting dilution. Ascites fluids from the positive subclones were prepared as described (Campbell, 1984). The ascites fluid used in these experiments contained a total of 30 mg/ml protein of which 50% was IgG, as judged by Coomassie Blue staining of SDS polyacrylamide gels.

**Immunocytchemistry and Toluidine Blue Staining**

Indirect immunofluorescence on MDCK cells grown on coverslips was performed as described (Louverd, 1980; Matlin et al., 1981). Immunofluorescence staining of MDCK cells grown on nitrocellulose filters was done using published procedures (Balarova-Stander et al., 1984; Fuller et al., 1984). Monoclonal antibody binding was also localized in semithin frozen sections of MDCK cells grown on nitrocellulose filters with indirect immunofluorescence as described by Tokuyasu (1973). Rhodaminated rabbit anti-mouse IgG and rhodaminated goat anti-rabbit Ig were used to stain the mouse monoclonal antibodies and the rabbit anti-guinea pig antibody, respectively. To stain the guinea pig antibody, a two-step procedure, first with rabbit anti-guinea pig Ig and then with rhodaminated goat anti-rabbit Ig, was employed.

Cell monolayers on filters to be stained with toluidine blue were fixed overnight or longer at 4°C in 3% paraformaldehyde in either PBS' or PBS. Fixed filters were rinsed once in PBS and stained for 1 h in 5 ml of an aqueous solution of 1% toluidine blue (Merck) and 1% NaBF₄ × 10 H₂O (pH not adjusted). The stained filters were washed in four changes of 5 ml PBS for 3 min each. They were then incubated for 3 min in 70% ethanol and then for 3 min in 100% ethanol. The dehydrated filters were then incubated in xylene until they became translucent and mounted between a glass slide and coverslip in Eukitt (Vitromed, Basel). The cells could be observed by light microscopy the next day after the Eukitt hardened.

**Immunoblotting and Gel Electrophoresis**

SDS extracts of MDCK cells were prepared as described (Matlin and Simons, 1983). Triton X-114 extraction of MDCK cells and phase separation was carried out as described (Bordier, 1981). A crude microsome fraction was prepared from strain 1 MDCK cells that were harvested as described above for immunizations. Cells were homogenized in 0.25 M sucrose, 10 mM Tris pH 7.6, 1 mM MgCl₂, containing 1 mM phenylmethylsulfonyl fluoride and 1 mM iodoacetamide with a special ball homogenizer (Balch et al., 1984). Enough strokes (5–10) were used to break >50% of the cells while leaving >90% of the nuclei intact. The nuclei and cell debris were removed by centrifugation at 10,000 rpm for 30 min at a Ti 75 rotor (Beckman Instruments Inc., Palo Alto, CA). The pellet was resuspended in 200 μl homogenization buffer and mixed with 200 μl of 50 mM NaHCO₃, pH 11.0, for 20 min on ice. Membranes were pelleted at 45,000 rpm for 30 min in an SW 50.1 rotor (Beckman Instruments Inc.). The pellet was dissolved directly in gel sample buffer. The supernatant was first precipitated with 10% trichloroacetic acid using 100 μg RNA as carrier.

All gel samples were dissolved in SDS, reduced, and alkylated as described (Matlin and Simons, 1983). Polycrylamide gels containing 9% acrylamide were prepared as described (Piccioni et al., 1982) and run using the buffer system of Laemmli (1970). When gels were prepared for fluorography, they were fixed in 10% trichloroacetic acid at room temperature for 30 min, treated with En3Hance (Amersham, Buckinghamshire, England), vacuum dried, and exposed to X-OMAT-AR film (Eastman Kodak Co., Rochester, NY). Immunoblotting was done as described (Burke et al., 1982). A 1:10,000 dilution of r1 ascites fluid and a 1:1,000 dilution of rabbit anti-mouse Ig labeled with peroxidase (Institut Pasteur, Paris) were used to stain the blots.

**Preparation of Labeled Trypsin Digests and Immunoprecipitation**

Strain 1 cells were grown for 2 d in 75-cm² flasks. Each flask was labeled for 15–20 h with 0.5 mCi [³⁵S]methionine in 5 ml of methionine-free MEM containing 10% fetal calf serum. The cells were rinsed five times in digestion buffer (140 mM NaCl, 4.7 mM KCl, 10 mM Hepes, pH 7.4, 1.2 mM CaCl₂, 0.6 mM MgCl₂, 0.1 mM Na deoxycholate, 0.2% SDS, 0.15 M NaCl, 20 mM Hepes, pH 7.5, and once with distilled H₂O and were eluted from the Pansorbin by boiling for 3 min in 100% ethanol. The dehydrated filters were then incubated in xylene
Antibodies to MDCK Surface Proteins

Rabbit anti-uvomorulin (serum 1) and rabbit anti-leucine aminopeptidase were gifts of Daniel Louvard (Institut Pasteur, Paris). Rabbit anti-uvomorulin Fab (serum 2) and the rat monoclonal antibody to uvomorulin were generous gifts of Dietmar Vestweber and Rolf Kemler (Max Planck Institute, Tübingen, FRG). Beat Imhof and Walter Birchmeier (Tübingen) provided the anti-Arc-1 monoclonal antibody, and Pamela Cowan (German Cancer Research Center, Heidelberg, FRG) provided the guinea pig anti-desmosomal glycoprotein 4 antiserum. The monoclonal anti-38K and the monoclonal anti-114K antibodies have been described (Balcarova-Stander et al., 1984). Monoclonal antibody A.7A5 was obtained in the hybridoma fusion described in this paper.

Results

Reversible Resealing of Tight Junctions Depends on Surface Protein(s)

The strain I MDCK cells used for this assay exhibit a higher transmonolayer resistance when grown on nitrocellulose filters than strain II MDCK cells—2,000–10,000 ohm cm² as compared with 100–300 ohm cm² (Richardson et al., 1981). Measured values of resistance are more variable for high-resistance epithelia. Leaks in very small fractional areas of the epithelium, such as those that occur at the monolayer edge, cause large decreases in measured transepithelial resistance (Diamond, 1977). In our experience with strain I MDCK cells, there was always more variability in transmonolayer resistance at higher values, especially those >2,000 ohm cm² (Fig. 1).

The tight junctions of strain I MDCK cells, like those of strain II MDCK (Martinez-Palomo et al., 1980), could be reversibly opened by manipulating extracellular Ca²⁺ levels. When strain I MDCK monolayers were incubated in Ca²⁺-free medium at 37°C, the transmonolayer resistance fell rapidly (Fig. 1). Within 15 to 20 min of incubation the resistance dropped ~100-fold to the value of a bare nitrocellulose filter measured in our system, i.e., ~40 ohm cm². The opening of intercellular junctions was apparent to some extent by light microscopy when monolayers on filters were stained with toluidine blue (not shown). If Ca²⁺ ions were restored within 15–20 min from the start of the Ca²⁺-free incubation and incubation was continued at 37°C, the transmonolayer resistance recovered rapidly (Fig. 1). The resistance recovered nearly to starting values over a 30 to 60-min period. However, when the Ca²⁺-free incubation was extended (40 min in Fig. 1), the resistance recovered much more slowly. Martinez-Palomo et al. (1980) described a similar decrease in the rate of resistance recovery of the strain II MDCK cells after prolonged incubation in Ca²⁺-free media. The basis of this loss of recoverability after prolonged Ca²⁺-free incubations is not known. It could have been due to either a much more extensive disassembly of intercellular junctions or to some unrelated disruptive effect of low Ca²⁺ treatment that developed with a slower time course.

It was necessary to optimize conditions to make it possible to use the rate of recovery of transepithelial resistance as an assay of the ability of the cells to reform tight junctions. For example, in the experiment shown in Fig. 1, incubation at 37°C for exactly 17 min in the Ca²⁺-free medium gave complete loss of electrical resistance while enabling extensive and rapid recovery upon Ca²⁺ restoration. Optimal timing of this incubation period was critical to within minutes (see below). Only monolayers with starting transepithelial resistances of >2,500 ohm cm² were used in all experiments. As long as the starting resistance of the monolayer was >2,500 ohm cm², the initial rate of recovery of resistance was independent of the starting resistance (not shown). One additional requirement to maintain or to recover high transmonolayer resistance was the inclusion of 1.0% fetal calf serum in the incubation medium. Omitting the serum caused a loss of resistance at 37°C even in the presence of Ca²⁺ to ~200 ohm cm² over a 30 to 60-min period.

To assay the effects of agents such as proteases or antibodies on the ability of monolayers to re-form tight junctions, an incubation period between the Ca²⁺-free opening phase and the recovery phase was required. To this end, an intermediate incubation period at reduced temperature was included. When the recovery incubation was carried out at 22 instead of 37°C, the rate of recovery was reduced more than fivefold (not shown). Upon return to 37°C with Ca²⁺, rapid recovery of resistance ensued. In this way, the cells could be treated with an agent for a 20-min intermediate period at room temperature even in the presence of Ca²⁺ before the intercellular junctions had time to reseal. The effect of the agent was then assayed by subsequent incubation with Ca²⁺ at 37°C.

The sensitivity of transepithelial resistance recovery to trypsin treatment is shown in Fig. 2. Monolayers whose intercellular junctions had been opened in Ca²⁺-free medium were exposed to 200 µg/ml trypsin in PBS for 20 min at 22°C. The transmonolayer resistance recovered poorly during subsequent incubation with Ca²⁺ at 37°C as compared with mock handled controls. The effect of trypsin treatment (which was significantly milder than conditions used to passage MDCK cells) could not be explained by a loss of cells from the filter. The entire monolayer was inspected by light microscopy after toluidine blue staining. No obvious losses of cells from the filter were observed after trypsin treatment (not shown). Also, in one experiment the cells released from the filter into the
media during trypsinization and recovery were counted. Trypsin treatment caused no increase in the number of cells released from the filter as compared with controls, i.e., only 0.5% of the cells were released in both cases. Therefore, mild trypsinization of the cells probably cleaved or degraded some cell surface protein(s) required for the reformation of intercellular junctions that lead to the recovery of transepithelial resistance. This result confirmed the findings of Griep et al. (1983) that trypsinized and freshly passaged MDCK cells required protein synthesis to redevelop transmonolayer resistance. The trypsin sensitivity also demonstrated the feasibility of using this resistance recovery assay to identify surface proteins involved in the formation of epithelial junctions and the establishment of a functional occluding barrier.

**Antibodies to the Cell Surface Specifically Inhibit Resistance Recovery**

Several polyclonal rabbit antisera raised against whole MDCK cells (anti-MDCK) inhibited the redevelopment of transmonolayer electrical resistance. To test the effects of various antisera in the resistance recovery assay, dilutions of heat-inactivated antisera in recovery medium were added to the apical side of MDCK monolayers for the 20-min intermediate incubation at room temperature as described above. Fig. 3A shows the effect of IgG purified from one active anti-MDCK antisera on the recovery of transmonolayer resistance during a subsequent 37°C incubation with Ca²⁺. High concentrations of anti-MDCK IgG (4.4 mg/ml) completely inhibited the recovery of transmonolayer resistance over a 90-min recovery period. Greater dilutions of this IgG slowed the rate of resistance recovery in a dose-dependent manner. IgG from preimmune serum at high concentrations (4.0 mg/ml) had no effect on the rate of resistance recovery. The rate of recovery after treatment with the anti-MDCK IgG at the lowest concentration tested (0.55 mg/ml) was discernably different from the preimmune control. To obtain the effect with the anti-MDCK antisera as shown in Fig. 3, it was necessary to optimize carefully the time of incubation in the Ca²⁺-free medium before antibody treatment. If the junction opening time was too long, the rate of resistance recovery was poor even for the controls (Fig. 1). If the junction opening time was too brief, the recovery was rapid even in the presence of active anti-MDCK sera (not shown). Presumably the intercellular junctions needed to be opened sufficiently to allow access and binding of antibodies to enough sites to inhibit subsequent junction reformation. Optimal timing varied slightly between batches of filter-grown monolayers from ~15 to 18 min. Therefore, it was often necessary to run a pretrial to optimize the timing for a given set of filters.

The effect of the anti-MDCK IgG could not be attributed to a nonspecific generalized cross-linking of antigens of the cell surface. Fig. 3B shows that monovalent Fab' fragments prepared from anti-MDCK IgG also strongly slowed the recovery of transmonolayer resistance. Since Fab' fragments cannot cross-link antigens, their inhibition of resistance recovery must have resulted directly from their binding to cell surface antigens.
It is unsurprising that the anti-MDCK sera, having been raised against entire MDCK cells, stained the entire MDCK cell surface brightly by immunofluorescence and probably reacted with many different cell surface antigens. The functional activity in the resistance recovery assay, however, appeared to be due to specific components of the antisera. Several antisera that bound to the surface of MDCK cells by the criterion of immunofluorescent staining, failed to inhibit the recovery of transmonolayer resistance. Fig. 4 shows the staining of MDCK with several different antisera that were tested in the assay. The antiserum shown in Fig. 4A stained MDCK cells very brightly at high dilution (1:500). It was an anti-MDCK serum taken from an earlier boost than the active anti-MDCK serum shown in Fig. 3. Yet, this antiserum had no detectable effect in the resistance assay even at a 1:3 dilution (Table I). Thus, the titer of the anti-MDCK sera for surface binding was much higher and developed sooner during immunization than the titers for functional activity. Several antibodies directed against specific surface proteins of MDCK cells also failed to inhibit the recovery of transmonolayer resistance (Table I). These antibodies (except A.7A5) were tested in the functional assay at concentrations 4-10-fold greater than those required to stain the cell surface intensely by immunofluorescence (Fig. 4). The functionally ineffective antibodies included some directed against apical membrane proteins (Fig. 4, B and C) and some directed against basolateral membrane proteins (Fig. 4, D–F). These results showed that the ability of the MDCK cells to recover transepithelial resistance was quite vigorous and was not inhibited simply by the binding of IgG to various surface proteins. The inhibition caused by the active anti-MDCK IgG shown in Fig. 2 probably was due to their binding to some specific subset of cell surface antigens.

**Generation of a Monoclonal Antibody that Inhibits the Resealing of Tight Junctions**

We used a monoclonal antibody approach to identify individual antibodies that would inhibit the recovery of transepithelial

| Antibody                  | Recovery of resistance (% control at 30–45 min) | n  |
|---------------------------|--------------------------------------------------|----|
| Anti-MDCK (first boost)   | 113 ± 10                                         | 5  |
| Anti-aminopeptidase       | 79 ± 25                                          | 3  |
| Anti-114K, apical         | 109 ± 24                                         | 3  |
| Anti-58K, basolateral     | 122 ± 23                                         | 3  |
| Anti-desmosomal gp4       | 108 ± 13                                         | 8  |
| Hybridoma A.7A5           | 98 ± 13                                          | 3  |

Antibodies were added for a 20-min intermediate incubation as in Fig. 3. Results are shown at 30 to 45 min recovery time because this was found to be the most reliable yet sensitive recovery interval. Values shown are the mean ± SEM. In these experiments, the active anti-MDCK antisera inhibited the recovery of resistance to <30% of control at this interval with a threefold dilution. The antibody dilutions used were anti-MDCK (taken after first boost), 1:3 serum; anti-aminopeptidase, 1:20 serum; anti-114K, 1:10 ascites; anti-58K, 1:10 ascites; anti-desmosomal gp4, 1:25 serum; hybridoma A.7A5, undiluted.

*Table 1. Lack of Inhibition of Resistance Recovery by Antibodies to Several Cell Surface Proteins*

![Figure 4. Immunofluorescence staining of the surface of strain I MDCK with various test antibodies. MDCK cells were grown on glass coverslips. In A–C, cells were fixed and washed in Ca²⁺, Mg²⁺-containing PBS before antibody staining. In D–F, cells were treated with PBS containing 2.5 mM EGTA for 7 min at 37°C before fixation in order to open the tight junctions. (A) 1:500 dilution of anti-MDCK serum taken after the first boost. (B) 1:100 dilution of anti-leucine aminopeptidase. (C) 1:100 dilution of ascites fluid monoclonal antibody, anti-114K. (D) 1:30 dilution of ascites fluid monoclonal antibody, anti-58K. (E) 1:100 dilution anti-desmosomal glycoprotein 4. (F) Undiluted hybridoma supernatant, A.7A5. Bar, 20 μm.*
racial resistance. To screen large numbers of hybridoma supernatants it was necessary to modify the resistance assay. To this end, the strain I MDCK cells were grown in individual wells of 96-well Millititer plates that have nitrocellulose filters on the bottom. Monolayers grown in these wells exhibited reliably transepithelial resistances of >2,500 ohm·cm². The individual wells were electrically isolated from one another, i.e., neighboring wells with and without cells had no influence on each other. The functionally active polyclonal anti-MDCK antibody served as an important tool to adapt the assay conditions to the Millititer plates. In fact, because of the sensitivity of the assay to the time of the Ca²⁺-free incubation, it was necessary to include the active polyclonal antibody as a positive control in every screen. This ensured that no positive hybridomas were missed due to variations in the timing of the assay.

One monoclonal antibody was obtained from the first hybridoma fusion that strongly inhibited the recovery of transepithelial electrical resistance. In this fusion only one positive hybridoma supernatant was detected out of 209 tested. However, many of the 209 that were negative could be shown to stain the MDCK cell surface (for example, Fig. 4F). The culture supernatant of the positive hybridoma remained active after it had been cloned by limiting dilution (Fig. 5A). The monoclonal antibody produced by this hybridoma line (subclass IgG₁) was named rrl. Given the specificity of antibody effects in this assay and the low frequency of positive hybridoma supernatants, it is very likely that antibody rrl recognizes an antigen on the cell surface that is functionally important in the reformation of the epithelial occluding barrier.

To study the kinetics of the inhibition of resistance recovery, rrl was assayed using large filters in mini-Marbrook chambers (Fig. 5B). An rrl ascites fluid (containing 15 mg/ml IgG) was tested at two dilutions. A 1:100 dilution inhibited the recovery of resistance strongly, to the same extent as very high concentrations of the active anti-MDCK polyclonal antisera. A 10-fold decrease in rrl concentration inhibited resistance recovery significantly but less strongly and was largely overcome at long recovery times. There was some recovery of transeptal interlayer resistance at long times even with higher concentrations of rrl antibody. This remaining long-term recovery could have resulted simply from the experimental procedure used, i.e., due to the short opening times in Ca²⁺-free medium or short period of treatment with antibody. Alternative explanations cannot yet be ruled out. New antigens may have been brought to the surface as a result of new synthesis or membrane recycling, or tight junctions may have begun to re-form even when all of the surface antigens were completely blocked with antibody.

The antigen recognized by the monoclonal antibody rrl was localized to the basolateral surfaces of MDCK cells by immunofluorescence staining. The antibody failed to stain the apical cell surface of confluent monolayers when the intercellular junctions were kept intact (Fig. 6, A and C). When the tight junctions were opened by treatment with EGTA before fixation, a ring of fluorescent staining around the cell periphery was apparent (Fig. 6B). Access of the antibody to the antigen on the basolateral surfaces did not require that the cell junctions had been opened by EGTA treatment. It was possible to stain some of the antigen on the basolateral surface of confluent cell monolayers that had intact junctions by applying the antibody directly to the basolateral surface through the nitrocellulose filter (Fig. 6D). To determine the distribution of the total cellular antigen, cell membranes were permeabilized with 0.2% Triton X-100 before antibody staining (Fig. 6E). The staining appeared much more intense but remained restricted to the lateral cell periphery. No evidence for intracellular pools of antigen was apparent at this level of resolution. Thus, the antigen recognized by monoclonal antibody rrl is a surface component of the basolateral plasma membrane of MDCK cells.

A better view of the polar distribution of the antigen recognized by rrl was obtained by staining semithin frozen sections of the cell monolayer which were cut perpendicular to the plane of the substratum (Fig. 7). The antigen recognized by rrl seemed to be present mostly in the lateral plasma membrane where the cells come into contact. However, it
Figure 6. Immunofluorescence localization of the rrl antigen in MDCK strain 1 cells. In A and B cells were grown on glass coverslips. In C-E cells were grown on nitrocellulose filters in mini-Marbrook chambers. (A) Cells were washed and fixed in PBS containing Ca\(^{2+}\) and Mg\(^{2+}\). (B) Cells were treated with PBS\(^{-}\) containing 2.5 mM EGTA for 7 min at 37°C before fixation to open the junctions prior to staining. (C-E) Fixation and washes all contained Ca\(^{2+}\) and Mg\(^{2+}\). (C) Staining with antibodies from the apical side. (D) Staining with antibodies from the basolateral side through the filter. (E) Cells were permeabilized with 0.2% Triton X-100 for 5 min after fixation but before antibody staining. Bar, 20 μm.

was not possible to tell at this level of resolution whether the antigen was more concentrated in the lateral membrane between cells than in the basal membrane which contacts the substratum. The antigen did not appear to be highly localized to any particular junctional element or position along the lateral plasma membrane.

Antibody rrl recognized a single polypeptide of 118 kD on Western blots of total MDCK cellular extracts (Fig. 8, lane a). The nature of the association of this polypeptide with the membrane was characterized preliminarily (Fig. 8, lanes c-g). Alkaline extraction preferentially removes peripheral

Figure 7. Immunofluorescent localization of the rrl antigen on frozen sections of strain 1 MDCK monolayers. Strain 1 MDCK cells were grown on nitrocellulose filters. Monolayers were fixed and frozen, and 1-μm sections were cut. (A) Staining with antibody rrl. (B) Phase-contrast image of the same field. Bar, 20 μm.
membrane proteins, leaving integral membrane proteins associated with the lipid bilayer (Hubbard and Ma, 1983). Crude cell membranes prepared from MDCK cells were extracted with a pH 11.0 bicarbonate solution and then centrifuged. The detergent extract was separated into two phases by warming to 30°C and then centrifugation. Lane d, aqueous phase supernatant; lane e, detergent pellet. Lanes c-f were all incubated together in antibody rrl. Binding of the monoclonal antibody was visualized by peroxidase reaction after incubation in a peroxidase-coupled rabbit anti-mouse IgG.

The 118-kD Polypeptide is Related to Uvomorulin

A number of Ca²⁺-dependent cell adhesion molecules have been reported in several systems which have a molecular weight of ~120,000. These include uvomorulin (Hyafil et al., 1981), cadherin (Yoshida-Noro et al., 1984), L-CAM (Gallin et al., 1983), and CAM 120/80 (Damsky et al., 1983), which may all be the same molecule or a related set of adhesion molecules (Edelman, 1984). Because our monoclonal antibody inhibited a Ca²⁺-dependent cell interaction and recognized a 118-kD polypeptide, we wished to compare this polypeptide to the uvomorulin, L-CAM class of cell adhesion molecules.

A common characteristic of all of these related cell adhesion molecules is that they can be cleaved from the cell surface in the form of an 81-kD polypeptide by trypsin in the presence of Ca²⁺. Fig. 9 shows that antibody rrl also recognized the same trypsin fragment. A trypsin digest of [³⁵S]methionine-labeled MDCK strain I cells was immunoprecipitated with a monoclonal antibody to uvomorulin (Fig. 9, lane a) and with antibody rrl (lane b). Both antibodies immunoprecipitated a polypeptide of identical mobility on SDS gels. The antibodies also immunoprecipitated major polypeptides of identical mobility (118 kD) from detergent lysates of cells (not shown). To determine whether the antibodies recognized exactly the same polypeptides, a sequential immunoprecipitation was carried out (Fig. 9, lanes a-c, first round; lanes d-f, second round). When the 81-kD polypeptide released by trypsinization was first removed by immunoprecipitation with anti-uvomorulin (Fig. 9, lane a), it no longer could be precipitated by antibody rrl (lane d). In contrast, when no antibody was included in the first round (Fig. 9, lane c) or when removal with antibody rrl was only partial (lane b), the 81-kD polypeptide was immunoprecipitated by antibody rrl in the second round (Fig. 9, lanes f and e, respectively). Thus, rrl and anti-uvomorulin competed for the same 81-kD polypeptide released from MDCK cells by trypsinization. They therefore recognize at least one polypeptide in common in MDCK cells.

We also tested whether several different antibodies to uvomorulin and to a uvomorulin-like polypeptide that were
The resistance recovery assay monitors as its endpoint, the integrity of the zonula occludens of a monolayer of epithelial cells. The measurement of transepithelial electrical resistance has been routinely used to characterize the tightness of various transporting epithelia (Claude, 1978; Diamond, 1977; Marcial et al., 1984; Martinez-Palomo et al., 1980). For our assay, we have chosen a cell line, MDCK strain I, which has a high transepithelial resistance, i.e., >2,000 ohm·cm² (Fuller et al., 1984; Richardson et al., 1981). Transepithelial resistances of these cells have been used as a standard in the measurement of transepithelial electrical resistance. The Ca²⁺-dependent opening of tight junctions in these cells has been used as a model system for the study of the mechanism underlying the reversible Ca²⁺-dependent opening of the tight junctions. The assay developed here measures the recovery of the transepithelial resistance after exposure to various agents that disrupt the cell monolayer. The recovery of transepithelial resistance is monitored by measuring the resistance of the cell monolayer before and after exposure to the agent. The resistance recovery assay has been optimized to minimize the possible perturbation of the cell monolayer. It begins with confluent, polarized monolayers of MDCK cells that have well-developed intercellular junctions. The Ca²⁺ ions are removed for the shortest time possible to allow access of reagents to the cell membrane while maximizing the ability of the junctions to recover. The conditions of short Ca²⁺-free incubation used in this assay probably avoids gross disruption of cells from their monolayer configuration. In contrast, many assays for the identification of cell adhesion molecules have employed completely dissociated cells (Edelman, 1984). An attempt has been made here to optimize the resistance recovery assay in order to study the events most closely associated, at least in time, to the reformation of the tight junctions. Thus, any molecules specifically identified with this assay can be considered to participate intimately in tight junction formation.

To identify specific molecules involved in tight junction assembly, we have looked for antibodies that block the recovery of transepithelial resistance. Such an immunological approach has been used successfully to identify several different cell adhesion molecules (Müller and Gerisch, 1978; Edelman, 1984) as well as specific molecules involved in immune recognition such as the antigen specific T cell receptor (Haskins et al., 1983). The specificity of the antibody inhibition approach in this assay was demonstrated because a variety of antibodies that bind to the surface of MDCK cells did not inhibit the recovery of transmonolayer resistance. Simple steric hindrance between apposing lateral cell membranes for example, could not account for the inhibition caused by the active antibodies. The active polyclonal antisera raised against the entire MDCK cell, although of low titer functionally and of low biochemical specificity, demonstrated the feasibility of finding specific active antibodies and were essential for working out a rapid assay to screen for monoclonal antibodies. One monoclonal antibody, rrl, has been obtained so far that strongly inhibits the recovery of transepithelial resistance. It should be possible to use this assay to obtain additional monoclonal antibodies that inhibit tight junction formation.

The monoclonal antibody rrl that was identified by the resistance assay recognizes a 118-kD polypeptide that is the same as or related to the cell adhesion molecules uvomorulin.
or L-CAM. All of these polypeptides have a similar molecular weight on SDS gels, and they all can be released from the cell surface as 81-kD fragments by trypsin digestion in the presence of Ca^{2+} (Damsky et al., 1983; Gallin et al., 1983; Hyafil et al., 1981; Behrens et al., 1983) (Figs. 8 and 9). Definitive evidence that the r1 antibody reacted with an MDCK form of uvomorulin was obtained by sequential immunoprecipitation. A monoclonal antibody raised to mouse uvomorulin and antibody r11 competed for the same 81-kD trypsin fragment.

The trypsin sensitivity of the MDCK uvo- morulin-like polypeptide could account for the sensitivity of the recovery of electrical resistance to trypsin shown in Fig. 2. The uvo- morulin-dependent cell interaction could also be partially if not completely responsible for the sensitivity of junction opening to Ca^{2+} removal. Uvomorulin and L-CAM have been identified as mediating Ca^{2+}-dependent cell adhesion events and have been shown to exhibit a Ca^{2+}-dependent conformational change (Hyafil et al., 1981; Gallin et al., 1983).

The exact relationship between the MDCK protein recognized by antibody r1 and the authentic uvomorulin involved in early mouse embryo compaction remains to be clarified. Of the antibodies to uvomorulin tested in the resistance recovery assay, only one monoclonal to uvomorulin had an effect. It is not clear why most of these uvomorulin antibodies, which do bind to MDCK cells, were impotent in the resistance recovery assay. The trivial explanation is that their affinities for the MDCK form of uvomorulin are too low to be active in this assay. An interesting possibility is that the differences between the antibodies could be due to their binding to different functional domains or different molecular forms of the uvomorulin molecule. Uvomorulin may consist of a set of closely related molecules. Under appropriate conditions in some tissues, antibodies to uvomorulin recognize 100- and 92-kD polypeptides in addition to the 120-kD form (Gallin et al., 1983; Boller et al., 1985). In mouse embryonal carcinoma cells these forms seem to be distinct by criteria of peptide mapping (Nadine Peyrieras, personal communication). It is not yet certain which of these polypeptides are the relevant substrates for the blocking antibodies in the various assays. A more detailed comparison of the various monoclonal anti-uvomorulin antibodies as well as the characterization of the different uvomorulin or L-CAM forms and the gene(s) that encode(s) for them (Gallin et al., 1985) will be required to understand this system of cell adhesion molecules.

Our discovery that a monoclonal antibody that inhibits the re-formation of tight junctions recognizes a uvomorulin-like molecule in MDCK cells indicates that these proteins have important physiological roles in adult epithelia. Previously, the roles of uvomorulin and L-CAM have been defined and discussed primarily in an embryological context. Antibodies to uvomorulin block the compaction of early mouse embryos (Hyafil et al., 1981) and antibodies to L-CAM block the aggregation of embryonic liver cells (Gallin et al., 1983). Uvomorulin and L-CAM are widely distributed, however, in many adult epithelia (Thiery et al., 1984; Boller et al., 1985; Behrens et al., 1985). Also, antibodies to related molecules have been shown to act on epithelial cells in culture. CAM 120/80 causes the dissociation of human mammary tumor cells (Damsky et al., 1983), and anti-Arc-I dissociates low-resistance MDCK cells (Imhof et al., 1983). We find that an antibody to a uvomorulin-like molecule can act on a confluent, polarized epithelium of the high-resistance type to prevent tight junction reformation. It acts very rapidly and inhibits a cell interaction very closely associated in time with tight junction assembly. We propose therefore that uvomo- rulin-like proteins participate intimately in the establishment of the epithelial occluding barrier.

An important test of this proposed physiological role of uvomorulin-like proteins is whether it can be generalized to epithelia other than MDCK cell monolayers. The distribution of L-CAM in various embryological and adult epithelia has been interpreted exclusively in terms of its potential morpho- genetic and inductive functions (Edelman, 1984; Thiery et al., 1984). It will be informative to ask whether the expression of uvomorulin or its different molecular forms in various epithelial cells correlates with some physiological property of the epithelial occluding barrier. One such correlation has been reported (Behrens et al., 1985). A monoclonal antibody to a uvomorulin-like polypeptide, anti-Arc-I, strongly stained the epithelial cells of the kidney distal tubule, a tight high-resistance epithelium, whereas the cells of the proximal tubule, a leaky low resistance epithelium, seemed not to stain.

How might the uvomorulin-like proteins participate in the epithelial junctional complex toward the establishment of an occluding barrier? One possible insight comes from the observation that uvomorulin is localized to the zonula adherens of mouse intestinal cells (Boller et al., 1985). The zonula adherens circumscribes the cell just below the tight junction (Farquhar and Palade, 1963). Formation of the zonula adherens may be a prerequisite for the assembly of the tight junction. It is even possible that the zonula adherens in association with the actin-containing cytoskeleton (Volk and Geiger, 1984) is responsible for positioning the tight junction in its apical zone (Meza et al., 1980).

In contrast to its restricted localization in mouse intestinal cells, the uvomorulin-like molecule in MDCK cells seems to be distributed over the entire lateral plasma membrane (Fig. 8). This discrepancy may be attributable to differences in the organization of brush border-containing epithelium, such as the intestine, versus epithelia without brush borders, such as the MDCK cells. The cellular distribution of L-CAM has been reported to vary among different epithelial tissues (Thiery et al., 1984). The more diffuse distribution in MDCK cells suggests another interpretation of the role of uvomorulin in the formation of tight junctions. The Ca^{2+}-dependent adhe- sion mediated by uvomorulin may simply bring the lateral plasma membranes close enough together to allow the subse- quent assembly of the tight junctions. Further experiments with the high resistance strain I MDCK line should help elucidate the role of the uvomorulin-related molecule in the assembly of the tight junctions.

Uvomorulin may be just one of many proteins involved in the establishment of the epithelial occluding barrier. More screens for monoclonal antibodies in the resistance recovery assay described here may lead to the discovery of additional essential junctional molecules. It may even be a powerful approach toward identifying the structural components of the zonula occludens itself. We thank especially Annamette Ohlsen for performing the frozen section immunocytochemistry, Stephen Fuller for his helpful advice on the resistance measurements, Petra Riedinger for drawing the figures, Wendy Moses and Birgit Blanasch for patiently typing the manuscript, and Hilkka Virta for providing general laboratory sup-
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