Dissociation of Madin-Darby Canine Kidney Epithelial Cells by the Monoclonal Antibody Anti-Arc-1: Mechanistic Aspects and Identification of the Antigen as a Component Related to Uvomorulin

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ABSTRACT It has previously been shown that the monoclonal antibody anti-Arc-1 dissociates Madin-Darby canine kidney (MDCK) epithelial cells and changes their morphology in vitro (Imhof, B. A., H. P. Vollmers, S. L. Goodman, and W. Birchmeier, 1983, Cell, 35:667–675). In this article we demonstrate that the anti-Arc-1 antibody recognizes an uvomorulin-like molecule on MDCK cells, i.e., it immunoprecipitates an 84-kD protein fragment from a tryptic digest of cell surfaces in the presence of Ca$^{2+}$ (as does anti-uvomorulin antiserum). Furthermore, anti-uvomorulin antiserum prevents the binding of anti-Arc-1 to MDCK cells. The distribution of the Arc-1 antigen is also quite similar to that of uvomorulin: it is enriched at the cell–cell contacts both of MDCK cells and of cells in various canine tissues. In the intestinal epithelium the antigen could be further localized in the region of the junctional complex.

To study the mechanism of action of the dissociating antibody, MDCK cells grown on Nuclepore filters in Boyden chambers were exposed to anti-Arc-1 from either the upper or lower compartment. It could be shown that the antibody interfered with cell adhesion only from the basolateral but not from the apical cell surface. Antibody action was inhibited in the presence of colchicine but not cytochalasin B. Furthermore, cell dissociation was prevented when the cellular cAMP level was raised. These findings indicate that the anti-Arc-1 antibody acts on a target below the tight junctions (possibly on the antigen located in the junctional complex), and they confirm that cytoskeleton and metabolic factors are actively involved in the maintenance of junctional integrity.

In recent years the process of cell adhesion has been extensively studied. In one line of research, antibodies that specifically inhibit cell–cell contacts have been used to identify the components involved (1–6). The neural cell adhesion molecule (N-CAM) has been so identified, and it seems to form direct bridges between neural cells (1, 7, 8). Different types of adhesion components have been discovered in nonneuronal cells (liver cell adhesion molecule [L-CAM] in chickens [9]; uvomorulin and cadherin in the mouse [2, 10, 11]; and cell adhesion molecule 120/80 in humans [12]). Their biochemical characteristics and their distribution in different tissues indicate that these adhesion components may all be closely related. Thus, L-CAM/uvomorulin-like adhesion molecules are found on cell surfaces and have molecular weights of ∼120,000, from which 84,000-mol-wt fragments can be cleaved by trypsin in the presence of Ca$^{2+}$ (2, 13). They are enriched at cell contact areas in epithelial tissues (9), and uvomorulin has been localized by immunoelectron microscopy at the intermediate junctions of the intestinal epithelium.

Abbreviations used in this paper: Con A, concanavalin A; L-CAM, liver cell adhesion molecule; MDCK, Madin-Darby canine kidney.
(14). Antibodies against this group of adhesion molecules can disturb cell-cell adhesion in tissue culture and also interfere with the compaction of mouse embryonic blastocysts (2, 3).

Our laboratory recently described the monoclonal antibody anti-Arc-1, which dissociates Madin-Darby canine kidney (MDCK) epithelial cells in tissue culture (5). Untreated MDCK cells are structurally and functionally polarized, i.e., they exhibit tight intercellular junctions and can transport ions vectorially. The anti-Arc-1 antibody seems to abolish this polarity: the cells lose their intimate contacts, they reduce gap junctional communication, they become fibroblastic, and they elaborate microvilli on the lateral surfaces (5). In this article we show that the Arc-1 antigen is related to uvomorulin. It is concentrated at the junctional complex of the intestinal epithelium, and the anti-Arc-1 antibody immunoprecipitates an 84-kD trypsin fragment from MDCK cells. We further show that the antibody functionally acts from the basolateral but not the apical side of the cells and that its action is modified by drugs that alter cytoskeleton and cell metabolism.

MATERIALS AND METHODS

Immunofluorescence of Cells and Frozen Sections: MDCK cells were cultured to ~70% confluence on glass coverslips overnight (5), fixed with 3% formaldehyde in phosphate-buffered saline (pH 8.3) for 15 min, and permeabilized with 1% Triton X-100 for 5 min. They were then stained with 70 μg/ml purified anti-Arc-1 antibody (5), and appropriately diluted fluorescein-labeled rabbit anti-mouse immunoglobulin (Miles-Yeda, Rehovot, Israel) was added. All steps were carried out at room temperature. Specimens were mounted in Mowiol 4-88 ( Hoechst, Frankfurt) containing 1 mg/ml p-phenylenediamine (to reduce fluorescence quenching) and examined on a Leitz Orthoplan photomicroscope.

Cryostat sections were cut to thicknesses of 0.5 or 4-7 μm on a Sorvall ultracryotome MT-2B (DuPont Instruments-Sorvall Biomedical Div., Wilmington, DE) equipped with a frozen thin sectioning system or a Reichert-Jung Fricocut model 2700 (Jung, Nusseck, FRG). Sections were fixed with formaldehyde on gelatine-coated glass coverslips and prepared for immunofluorescence as described above.

Immunoprecipitation and Binding Experiments: MDCK cells were grown on 15-cm plates (Falcon Labware, Oxnard, CA) and labeled with [35S]methionine as previously described (15). The cells were scraped off the plates into 1 ml L-CAM assay buffer (13) containing Ca** and 0.2 mg/ml trypsin (type XI, Sigma Chemical Co., St. Louis, MO) and incubated for 40 min at 37°C. Egg-white trypsin inhibitor (0.8 mg, type II-O, Sigma Chemical Co.) was then added, and the cells were centrifuged for 5 min at 1000 g. The supernatants were then pelleted with 50 μl of a 10% suspension of Staphylococcus aureus, which had been presaturated with affinity-purified rabbit anti-mouse antibody. Antibody-Arc-1 antibody (20 μg), control IgG1 (20 μg anti-FC-1: see reference 16), or rabbit anti-uvomorulin serum (20 μg) was then added, and after 3 h on ice the immune complexes were pelleted with 50 μl S. aureus solution. For the precipitation of IgG1-antigen complexes, bacteria presaturated with rabbit anti-mouse immunoglobulin were used. The anti-uvomorulin serum was a generous gift of D. Vestweber and R. Kemler, Tübingen (17). After they were extensively washed with L-CAM assay buffer containing 0.1% Triton X-100, the bacteria were eluted with SDS-dissociation buffer at 95°C, and the extracts were analyzed by SDS-gel electrophoresis and autoradiography (15). Alternatively the trypsin extracts were adsorbed on a 0.4 ml concanavalin A (Con A)-Sepharose column (Pharmacia Fine Chemicals, Uppsala, Sweden) and eluted with 200 mM α-methyl-D-mannoside in L-CAM assay buffer. The eluates were then immunoprecipitated as described above.

Binding of [125I]-labeled anti-Arc-1 antibody to fixed and permeabilized MDCK cells was performed as described (5, 16). For competition experiments, rabbit anti-uvomorulin antisera or preimmune serum was added together with the radioactive antibody, and bound radioactivity was measured after 3 h.

Boydén Chamber Experiments: MDCK cells (5 x 10⁶) were grown overnight on gelatine-coated Nucleopore filters (13-μm diam, 0.05-μm pore) in modified Boyden chambers (0.8-cm well diam; Bio-Rad Laboratories, Richmond, CA) until confluence was reached. Anti-Arc-1 antibody (70 μg/ml in complete culture medium) was then added to either the upper or the lower chamber. After 3 h of further culture, cells were fixed with 2.5% glutaraldehyde and inspected for cell dissociation by light and scanning electron microscopy (5). For immunofluorescence, cells in the Boyden chamber were washed after short exposure to the anti-Arc-1 antibody, fixed with 3% formaldehyde, permeabilized with 1% Triton X-100, and incubated with fluorescein-labeled rabbit anti-mouse immunoglobulin as described above.

Drug Experiments: Cells grown on glass coverslips (~ ~70% confluence) were incubated with medium containing 50 μg/ml anti-Arc-1 antibody plus the various drugs at concentrations that alone did not markedly affect cell morphology: choliceine at 1 μg/ml; cytochalasin B at 0.05 μg/ml; 3-isobutyl-1-methylxanthine (18) at 50 μM; forskolin (19) at 50 μM; and NG̣ṆỌỌ-dibutyryl cAMP at 10 mM (all from Sigma Chemical Co.); A 23187 (20) at 10 μM (Calbiochem-Behring Corp., San Diego, CA); calmidazolium (21) at 15 μM (Boehringer Mannheim, FRG); and cycloheximide at 10 μg/ml. Concentrated stock solutions of the drugs were prepared in dimethyl sulfoxide except for choliceine and cycloheximide, which were in aqueous solution. Final concentrations of dimethyl sulfoxide (which alone had no effect on cell morphology or on antibody action) did not exceed 0.25%.

CAMP concentration was determined with a radioassay kit (Amersham International, Amersham, England) according to the manufacturer’s procedure. MDCK cells (2 x 10⁶) were fixed at 0°C in 0.1 M HCl, and the supernatants were lyophilized and analyzed for cAMP. Basal levels of cAMP were 4-6 pmol/mg cellular protein; forskolin increased this level by a factor of 25. Isobutyl methylxanthine by a factor of 2. Anti-Arc-1 had no measurable effect on the cAMP level.

Determination of Intercellular Communication: MDCK cells pretreated with various anti-Arc-1 and drug combinations (as described in Drug Experiments) were microinjected with lisamine rhodamine RB 200 (100 mM, Polysciences Inc., Warrington, PA) and incubated for 10 min at 37°C. For quantitation of cell coupling, 250 cells per experimental point were injected, and the microscope image was captured on a silicon intensifying camera and displayed on a TV monitor (22). Cells were considered to be coupled when their outlines were clearly visible in the fluorescent image (5).

RESULTS

Immunolocalization of the Arc-1 Antigen on MDCK Cells and Various Tissue Sections

To examine the location of the Arc-1 antigen, immunofluorescence was performed on cultured MDCK cells and on frozen sections of various canine tissues. No significant staining was observed if fixed MDCK cells were not permeabilized. However, detergent extraction resulted in a characteristic staining, often in a punctuate pattern, along the lines of cell-cell contacts (Fig. 1, a and b). A control monoclonal antibody of the same isotype showed only background staining. The presence of the antigen at cell-cell contacts was confirmed by immunofluorescence on tissue frozen sections. In the small intestine the antigen was restricted to the area of the junctional complex (Fig. 1, a and d). Again, no such staining was observed with the control monoclonal antibody. In the canine kidney cortex, Arc-1 was found at the epithelial cell-cell contacts of the distal tubules but was virtually absent from proximal tubules and glomeruli (Fig. 2, a and b). We could also detect the antigen on sections of the canine epidermis (Fig. 2c). Here the signal was confined to layers between the stratum corneum and the dermis. In the hair follicle Arc-1 was also restricted to a defined cell layer (Fig. 2d), the external root sheath. In the liver, the antigen was more uniformly distributed (Fig. 2e), but again was enriched at the cell-cell borders.

Relationship between anti-Arc-1 and the Cell-Adhesion Molecule Uvomorulin

Since the distribution of the Arc-1 antigen on canine tissue revealed some similarity to uvomorulin of the mouse system (14), we compared the two molecules by immunoprecipitation
FIGURE 1 Immunolocalization of the Arc-1 antigen on MDCK cells in tissue culture and on sections of the canine small intestine. (a) Immunofluorescence of fixed and permeabilized MDCK cells. (b) Corresponding phase-contrast image. (c) Immunofluorescence of the canine intestinal epithelium. (d) Corresponding phase-contrast image. The section was 0.5 μm thick. e, epithelial cell layer. lp, lamina propria. g, goblet cell. A control antibody (anti-FC-1, which does not react with MDCK cells; reference 16) showed no staining at the cell borders. (a and b) x 600. (c and d) x 770.

and in cell binding experiments. Uvomorulin can be specifically cleaved from the cell surface by trypsin in the presence of Ca²⁺, yielding a characteristic 84-kD glycoprotein fragment (2). Such a fragment could be immunoprecipitated from trypsin-treated MDCK cells by both anti-Arc-1 monoclonal antibody and a rabbit anti-uvomorulin serum (Fig. 3A). The 84-kD trypsin fragment of uvomorulin is a Con A-binding protein (2); we were also able to immunoprecipitate the 84-kD protein by the use of both antibodies from an eluant of a Con A column. In addition, anti-uvomorulin serum competed in a concentration-dependent manner with ¹²⁵I-anti-Arc-1 binding to fixed and permeabilized MDCK cells (Fig. 3B), whereas preimmune serum did not. These findings suggest that anti-Arc-1 recognizes an uvomorulin-like molecule in the canine epithelial cells.

Anti-Arc-1 Antibody Acts on the Basolateral but Not the Apical Side of MDCK Cells

The localization of the Arc-1 antigen at the junctional complex of the intestinal epithelium raised the question of from which surface the dissociating antibody might reach the functional target. The anti-Arc-1 antibody was therefore presented to either the upper or the lower surface of confluent MDCK monolayers growing on Nuclepore filters in Boyden chambers. Cell dissociation occurred only when the antibody was delivered to the basolateral side of the cells (Fig. 4, compare a with b). The average number of extended contacts of individual cells to their neighbors was 1.5 or 3.8, when the antibody was added to the lower or upper side, respectively (40 cells counted in representative fields). Apparently the tight junctions prevented the access of the antibody to its functional target when it was applied to the apical surface. This hypothesis was confirmed by immunofluorescence experiments using confluent, live MDCK cells in the Boyden chamber. The anti-Arc-1 antibody bound to the cell contact areas of living cells only when it was presented from the lower chamber (Fig. 4, compare c with d).

Modulation of Anti-Arc-1 Action by Drugs that Affect the Cytoskeleton and the cAMP System

The anti-Arc-1 antibody dissociates MDCK cell monolayers and thus induces pronounced morphological changes in the cells (Fig. 5, a and b; the average number of contacts between individual cells and their neighbors dropped from 4.2 to 1.4; see also above). Cytoskeletal components or metabolic events may contribute to these effects. To examine the involvement of the cytoskeleton, the influence of colchicine and cytochalasins on anti-Arc-1 activity was tested. In the presence of colchicine, disruption of the MDCK monolayer by anti-Arc-1 was inhibited (Fig. 5c; the average number of cell contacts was 3.9). Cytochalasin B did not inhibit but rather slightly promoted antibody action (Fig. 5d; the average number of contacts was 1.1; cytochalasin D had a similar effect). Apparently, an intact microtubular network is necessary for antibody activity whereas the microfilamentous network is less important.

We also tested the effect of various drugs that influence cell metabolism. Antibody action was prevented when cellular
cAMP was raised (Fig. 5, e and f; contact numbers 3.5 and 3.7). This was accomplished by the addition of isobutyl methylxanthine (which inhibits cyclic nucleotide phosphodiesterase [18]), or forskolin (which activates adenylate cyclase [19]; see also Materials and Methods), or dibutyryl cAMP. Compounds that affect other metabolic processes such as cycloheximide, calmidazolium (an inhibitor of calmodulin action [21]), the Ca\(^{2+}\) ionophore A 23187 (20), and serum (0–20%) did not interfere with the antibody effect (data not shown).

The anti-Arc-1 antibody also greatly reduces communication of MDCK cells through gap junctions, as assessed in dye coupling experiments (5). Most MDCK cells are coupled, and antibody treatment decreases the amount of coupling (Fig. 6, compare a with b). As observed for the morphological effects of the antibody, colchicine and the cAMP-elevating drug isobutyl methylxanthine counteract the uncoupling effects of the antibody (Fig. 6, c, e, and f), whereas cytochalasin B has no effect (Fig. 6 d). Under the conditions used, the drugs alone did not influence coupling.

**DISCUSSION**

In the present study we present evidence that suggests that the Arc-1 antigen of MDCK cells is an uvomorulin-like molecule. First, the anti-Arc-1 monoclonal antibody precipitates an 84-kD glycoprotein cleaved from the cell surface by trypsin in the presence of Ca\(^{2+}\). This cleavage product is characteristic of the L-CAM/uvomorulin family of adhesion proteins (2, 13). Second, anti-uvomorulin antiserum efficiently competes for the binding of anti-Arc-1 to its target on fixed and permeabilized MDCK cells. Third, both antigens are localized at the borders of epithelial cells in culture and in certain tissues. Last, Arc-1 and uvomorulin are restricted to the junctional complex of the intestinal epithelium. Taken together, these findings provide strong evidence for a structural and functional homology between Arc-1 and uvomorulin.

The intact epithelial cell adhesion molecules of the L-CAM/uvomorulin family are 120-kD proteins (3, 10, 13). In our earlier work (5) we identified Arc-1 as both 120- and 40-kD components by gel filtration of EDTA-cell extracts (uvomorulin can be extracted with EDTA from mouse teratocarcinoma cells [compare reference 10], although detergent extraction is currently preferred). Thus, the 120-kD Arc-1 might represent the intact adhesion molecule, whereas the 40-kD Arc-1 is probably a breakdown product. With the anti-Arc-1 monoclonal antibody we have so far been unable to immunoprecipitate the undigested 120-kD molecule from detergent extracts of MDCK cells. However, the 120-kD uvomorulin could be readily identified in MDCK cells after immunoprecipitation with anti-uvomorulin antiserum (not shown).
The Arc-1 antigen could be detected in a variety of canine organs such as skin, intestine, kidney, and liver. Despite this relatively widespread occurrence, it is restricted to specialized cell types within single tissues. For instance, it was detected on distal, but less on proximal, tubules of the kidney, on external but not internal root sheath of the hair follicle, and on distinct layers of the epidermis. The localization of the antigen in the distal kidney tubules correlates with the possible source of the MDCK cells (23), against which the antibody was raised. For L-CAM and uvomorulin, similar locations in certain epithelia have been reported. For instance, both proteins are enriched in the germinal layers of the epidermis and in the epithelia of the kidney tubules, but less in the epithelia of the glomeruli (9). The reason for this restriction in only some epithelia is not at present clear. It might reflect a common property of the various cell layers such as similar mechanical stability or similar state of differentiation.

We have also shown here that the Arc-1 antigen is enriched in the junctional complex of the intestinal epithelium (Fig. 1, c and d). This agrees with the distribution of uvomorulin in the intestine, where higher resolution analysis revealed yet further restriction to the intermediate junctions (14). We therefore could hypothesize that the anti-Arc-1 antibody produces cell dissociation by first binding to a target in the area of the junctional complex, e.g., at the intermediate junctions, thereby disturbing critical cell-cell contacts. Our Boyden chamber experiments support this assumption, since the cell-dissociating anti-Arc-1 antibody acts only from the basolateral and not from the apical side, which indicates that its functional target lies below the tight junctions. This also explains earlier observations from our laboratory, namely that cell dissociation by anti-Arc-1 was reduced when the MDCK cells grown on tissue culture plates were fully confluent; presumably the antibody could not penetrate the monolayer and reach its target in this case. In our earlier article (5) we showed that the Arc-1 antigen could also be detected on the apical surface of MDCK cells by the extremely sensitive technique of immunoscanning electron microscopy. The apical Arc-1 may thus represent a subpopulation of the antigen below the detection limit of immunofluorescence, or may only exist before the cells become fully polarized. Other investigators (24) have shown that polarization of certain antigens in MDCK cells does not occur until full confluence of the monolayer is reached.

The MDCK cell is an excellent in vitro system with which to study cell adhesion phenomena since it forms tight monolayers, is well polarized, and expresses a series of characteristic cell-cell and cell-substrate junctions (5, 25, 26). The present work indicates that the uvomorulin-like Arc-1 antigen represents a central adhesion component in the junctional complex of these cells, i.e., the disturbance of one type of adhesion molecule induces complete dissociation of the monolayer. Anti-Anc-1 destroys cell-cell contacts but also seems to abolish epithelial cell polarity, i.e., the cells move over one another and elaborate microvilli on their lateral surfaces (5). In effect, the cells are functionally deprived of Arc-1, as they would be after a deletion of the Arc-1 gene, or a down-regulation of the Arc-1 surface molecule. This suggests that the expression of Arc-1 is necessary for a correctly polarized monolayer and may alone be sufficient to impose polarity on the MDCK cells. If this is a correct model, then differential activation of genes for Arc-1-like molecules could be a decisive controlling factor.
factor during organogenesis, when numerous mesenchymal-epithelial transitions occur. Recently, a monoclonal antibody against mouse uvomorulin (DECMA-1) was obtained which also dissociates MDCK cells (Vestweber, D., and R. Kemler, manuscript submitted for publication). It will be interesting to find out whether this antibody also has free access to its target only from the basolateral cell surface, and whether other antibodies (e.g., anti-L-CAM) act in the same fashion on polarized epithelial cells.

How could the cytoskeleton- and metabolism-perturbing drugs interplay with the cell-dissociating antibody? It is clear that the dissociation and shape change of MDCK cells is a complex multistep process that shifts the cells from an epithelial towards a fibroblastic form (Fig. 5, a and b). In the epithelial state the MDCK cells are adhesive, communicative, and polar. In contrast, the fibroblastic cells exhibit little communication, few intercellular junctions, and a strong anterior-posterior polarity (25, 27). We can hypothesize that any modulator that shifts the cells between these two extreme states might also affect the degree of anti-Arc-1 action. For instance, it is known that cAMP can increase cell communication in certain systems (28), and colchicine has been shown to destroy anterior-posterior polarity of fibroblasts (29). Thus, under the influence of both these effectors an epithelial form might be favored. By contrast, cytochalasin B itself can induce spindle-shaped MDCK cells at higher concentration (e.g., at 10 μg/ml) and might therefore potentiate anti-Arc-1 action. After having considered the morphological changes during cell dissociation, we will now look at what might happen at the molecular level. The steric interference of the antibody with Arc-1 at or near cell contacts may lead to a cascade of further events, e.g., a local influx of Ca\(^{2+}\) followed by closure of the gap junctions (5, 30). The cells, having now broken off communications with their neighbors (Fig. 6), might then change their morphology, a change requiring cytoskeletal rearrangement. cAMP could counteract this since it keeps the gap junctions open (28) or might interfere with the cytoskeleton through phosphorylation. The colchicine experiments also point toward a critical role of the microtubules in these secondary processes.

We should also consider that the anti-Arc-1 antibody could actually trigger a change of differentiation of the MDCK cells from the epithelial to a fibroblastic (mesenchymal) stage. For instance, when Madin-Darby bovine kidney epithelial cells
FIGURE 5  Effect of the anti-Arc-1 antibody on cell dissociation; modulation by various drugs that affect cytoskeleton and cAMP metabolism. MDCK monolayers were incubated for 3 h with anti-Arc-1 antibody in the presence and absence of the drugs as described in Materials and Methods. Cells were used (a) without antibody and drugs, or with (b) anti-Arc-1 antibody alone, (c) anti-Arc-1 antibody plus colchicine, (d) anti-Arc-1 plus cytochalasin B, (e) anti-Arc-1 plus 3-isobutyl methylxanthine, or (f) anti-Arc-1 plus forskolin. Addition of dibutyril cAMP had the same effect as in e and f. Cells were processed for scanning electron microscopy as described in reference 5. Bar, 10 μm. X 900.
Figure 6 Effect of the anti-Arc-1 antibody on coupling of MDCK cells: modulation by drugs that affect cytokinin and cAMP metabolism. MDCK monolayers were incubated for 1.5 h with the anti-Arc-1 antibody in the presence and absence of the various drugs, which were used at the same concentrations as in Fig. 5. Cells were used (a) without antibody (control), or with (b) anti-Arc-1 antibody alone, (c) antibody plus colchicine, (d) antibody plus cytochalasin B, (e) antibody plus isobutyl methylyanthine, and (f) antibody plus isobutyl methylyanthine and cytochalasin B. After microinjection of lissamine rhodamine, the injected cells were grouped according to the number of neighboring cells that communicated with them (see reference 5).

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