Journal of Cell Science 104, 719-726 (1993)
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Elevated cAMP levels induce multilayering of MDCK cells without disrupting cell surface polarity

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SUMMARY

The effect of hormones on the morphology and cell surface polarity of the epithelial cell line MDCK was examined. When MDCK cells were seeded in high densities in media containing FCS a regular monolayer was formed. However, in serum-free medium supplemented with insulin, transferrin, prostaglandin E_1, hydrocortisone and triiodothyronine, the development of a multilayer with intercellular lumina was observed. In hormone-depletion studies we identified PGE_1 as the inducer of these multilayers. Since dibutyryl cyclic AMP and the phosphodiesterase inhibitor isobutyl methylxanthine could substitute for PGE_1, we conclude that an elevated intracellular cAMP level resulted in formation of the multilayer.

Further analysis by electron microscopy and immunocytochemistry revealed a polarized organization of the multilayered cells. Junctional complexes, enclosing microvilli-rich membrane domains, were found at the apices of adjacent cells facing the medium and those surrounding the intercellular lumina. Surprisingly, cells participitating in the formation of both the free surface and the surface of the intercellular lumen, exhibited two distinct membranes with microvilli, each separated by junctional complexes. Immunolocalization of membrane marker proteins demonstrated that an apical 114 kDa membrane protein was localized to the free cell surfaces, the same membrane domains where extensive microvilli were also observed. The distribution of a basolateral 58 kDa membrane protein was restricted to sites of cell contact. These results provided evidence that nontransformed epithelial MDCK cells form multilayers in response to elevated cAMP levels; however, they retain the potential of developing cell surface polarity.

Key words: cyclic AMP, epithelial polarity, MDCK cell multilayers

INTRODUCTION

Polarized epithelial cells form selective barriers between the compartments of an organism. To fulfil this function the plasma membrane is polarized into an apical and basolateral domain, which are each composed of different lipids and proteins (Simons and Fuller, 1985). Tight junctions prevent the paracellular diffusion of ions and macromolecules across the epithelium (Gumbiner, 1987). They also form a barrier to the intermixing of apical and basolateral membrane components by lateral diffusion (van Meer and Simons, 1986).

The polarized epithelial cell line MDCK has been widely used as a model system to study biogenesis of cell polarity and factors that result in the development of organized multicellular structures (Rodriguez-Boulan and Nelson, 1989). This cell line displays morphological and functional properties of kidney distal tubule cells (McRoberts et al., 1981). Analysis of the differentiation of MDCK cells suggests that the establishment of cell polarity is initiated by cell-cell and cell-substratum contact. Upon attachment of single MDCK cells to a substratum some apical membrane proteins become restricted to the free cell surface (Vega-Salas et al., 1987; Ojakian and Schwimmer, 1988). However, the formation of a basolateral membrane requires cell-cell contacts (Balcarova-Ständer et al., 1984; Vega-Salas et al., 1987).

Experiments with MDCK cells injected in chick embryos and mice have shown that these cells have retained the ability to form tubule-like structures (Leighton et al., 1970; Rindler et al., 1979). When the cells are grown within collagen gels the development of monolayered spherical cysts can be observed (Hall et al., 1982; Karst and Merker, 1988; Mangoo-Karim et al., 1989; Wang et al., 1990a,b). Furthermore, the culture of MDCK cells in the presence of fibroblast-conditioned medium within a collagen matrix has been found to induce epithelial morphogenesis of the cells into branching tubules, apparently due to the presence of the hepatocyte growth factor (HGF) (Montesano et al., 1991a,b). All these monolayer structures are well polarized,

with a smooth basal surface in contact with the basement membrane or the collagen gel and a microvilli-rich apical surface facing the lumen (Wang et al., 1990a; Montesano et al., 1991b). In contrast, when oncogenes are expressed in MDCK cells the cell-cell and cell-substratum contact is changed as well as the cell polarity (Warren and Nelson, 1987; Behrens et al., 1989; Schoenenberger et al., 1991; Schoenenberger and Matlin, 1991). In a recent study the transformation of MDCK cells with v-K-*ras* was found to cause multilayering and loss of apical polarity (Schoenenberger et al., 1991).

We report the formation of MDCK cell multilayers with intercellular lumina by increasing cAMP levels. The observed structures differ from multilayers developed upon oncogenic transformation, since the cells are closely attached to each other and to the filter support. Most importantly, the cells display a polarized organization, with apical membranes facing the free cell surfaces and the basolateral membranes at sites of cell contact. Our results indicate that cAMP-dependent changes in cell-cell adhesion lead to multilayering of MDCK cells without affecting their ability to polarize.

MATERIALS AND METHODS

MDCK cells (strain II) and the mouse monoclonal antibodies against the apical 114 kDa and the basolateral 58 kDa membrane proteins were gifts from K. Simons (European Molecular Biology Laboratory, Heidelberg, Germany). Media and reagents for cell culture were obtained from Biochrom, Berlin, Germany. Polycarbonate filters (Transwell TM 3412) were from Costar, Cambridge, MA. Hormones, dibutyryl cyclic AMP and isobutyl methylxanthine (IMBX) were obtained from Sigma, Deisenhofen, Germany.

Culture of MDCK cells on filters

MDCK cells were seeded at a density of 2×10^6 cells/cm^2 on 0.4 µm pore size polycarbonate filters and cultured in a 1:1 mixture of serum-free Dulbecco's modified Eagle's medium and Ham's F12. As serum supplements we used prostaglandin E_1 (25 ng/ml), transferrin (5 µg/ml), insulin (5 µg/ml), triiodothyronine (5×10^{-12} M) and hydrocortisone (5×10^{-8} M). To determine the effect of PGE_1 we used dibutyryl cyclic AMP (0.5 mM) and isobutyl methylxanthine (0.5 mM).

Preparation of cells for microscopy

MDCK cells grown on polycarbonate filters were fixed by replacing the culture medium with the respective fixative.

For morphological investigations cells were fixed first with 2.5% glutardialdehyde in 0.1 mol/l sodium cacodylate buffer (3.5 h) and then fixed with 1% OsO_4 (1 h). After treatment with 2% uranyl acetate overnight cells were dehydrated by a series of increasing concentrations of ethanol, infiltrated with Spurr´s epoxy resin (Spurr, 1969) and then polymerized at 70°C. Thin sections were analyzed in a Philips EM 300 electron microscope after double-staining with uranyl acetate and lead citrate.

For light microscopic immunostaining MDCK cells were fixed (2 h) with paraformaldehyde-lysine-phosphate buffer (PLP) (McLean and Nakane, 1974), washed and incubated overnight in 2% glycine in PBS. The cells adhering to filters were enclosed in agar-agar, infiltrated with sucrose (up to about 40% for cryoprotection) and frozen. Cryosections (8-10 µm) were cut, thaw-mounted onto Alcian blue-coated glass slides, dried and stored in the cold until use.

For pre-embedding immunostaining cells were fixed in PLP or with 4% paraformaldehyde and 1% glutaraldehyde in PBS.

Immunostaining

Light microscopy

To differentially label apical and basolateral plasma membranes of MDCK cells specific monoclonal antibodies (mAbs) were used. The mouse IgG monoclonal antibody 114 was directed against an apical membrane protein whereas mouse IgG mAb 58 was directed against a protein in basolateral membranes of MDCK cells.

Cryosections of PLP-fixed MDCK cells were treated with 2% glycine/PBS, then with 1% BSA, 0.1% Triton X-100 plus 0.2% Tween 20 in PBS and then with PBS containing 0.1% BSA and 0.05% Tween 20. Sections were incubated with the specific mAbs for 90 min at room temperature, washed and then incubated with fluorescein (FITC)-coupled secondary goat anti-mouse IgG antibodies. Sections were analyzed in a Photomikroskop I (Zeiss) equipped with an epifluorescence device.

In some preparations sections were sequentially double immunostained. First, sections were immunostained with mAb 114 (to label apical membranes), photographed and then again immunolabeled with the other mAb 58 (for basolateral membranes) in order to demonstrate the relative position of both membrane types.

Electron microscopic immunolabeling

To demonstrate immunolabeling of plasma membranes at the electron microscopic level the pre-embedding immunostaining method was applied. For labeling sheets of MDCK cells fixed with either 4% paraformaldehyde/1% glutaraldehyde and separated from the filter membrane (for mAb 58) or with PLP were treated as for light microscopic immunolabeling. However, for demonstration of primary antibody binding sites peroxidase-conjugated secondary antibodies were applied. After colour development with diaminobenzidene (Graham and Karnovsky, 1966) cells were refixed with 1% glutaraldehyde/PBS and 1% OsO_4, dehydrated and embedded in Spurr's resin (Spurr, 1969).

RESULTS

Formation of MDCK cell multilayers with intercellular lumina in hormonally defined, serum-free medium

MDCK cells which were seeded at high density (2×10^6 cells/cm^2) on plastic Petri dishes exhibited a different morphology depending on the culture medium. When cells were plated in culture medium containing 5% fetal calf serum the excess cells came off and a monolayer was formed (Fig. 1A). In contrast, in serum-free medium supplemented with insulin, transferrin, prostaglandin E_1, hydrocortisone and triiodothyronine, almost all cells attached and formed multilayered structures which covered 20-30% of the surface area (Fig. 1B). When we used higher plating densities (5×10^6 cells/cm^2) this percentage reached almost 100% (Fig. 1C).

In order to provide an optimal supply of nutrients, the following ultrastructural analyses were performed with cells grown on a permeable support. When the cells were seeded on filters at high density (2×10^6 cells/cm^2) in culture medium containing 5% fetal calf serum a regular monolayer was formed with microvilli abundant on the apical

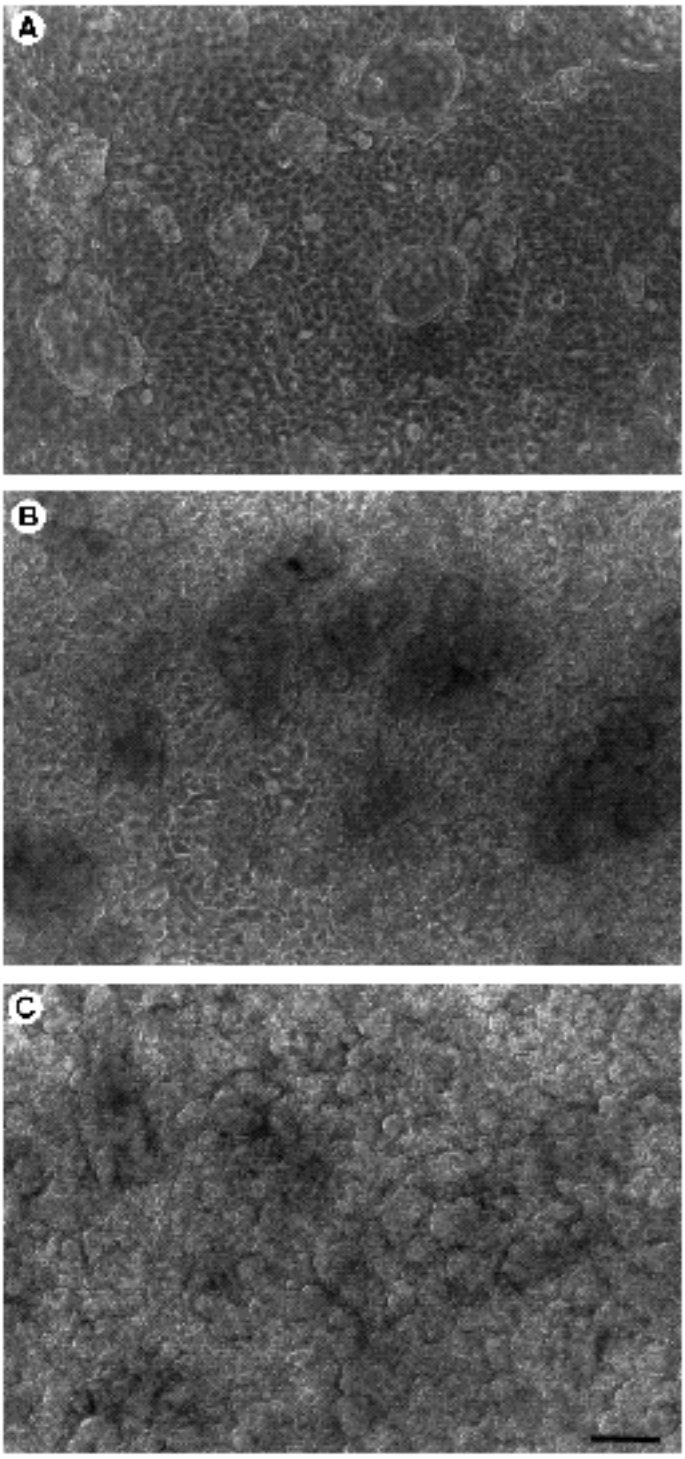

Fig. 1. Phase-contrast micrographs of MDCK cells cultured on plastic Petri dishes in media with FCS or in serum-free, hormone-supplemented media. MDCK cells were seeded on plastic petri dishes at a high plating density of (A,B) 2×10^6 cells/cm^2 or (C) 5×10^6 cells/cm^2 and cultured for four days in media containing (A) 5 % FCS or (B,C) serum-free media supplemented with insulin, transferrin, PGE_1, hydrocortisone and triiodothyronine. Cells grown as a monolayer (A) form domes which are typical of confluent MDCK monolayers on plastic substrata. Bar, 100 µm.

surface (Fig. 2A). Individual cells were cuboidal and the nuclei were located basally with the Golgi apparatus in a supranuclear position. Junctional complexes occurred at contact sites near the apical plane of neighbouring cells. In contrast, when grown in serum-free medium supplemented with hormones, the development of a multilayer with intercellular lumina could be observed (Fig. 2B). Cell size and cell shape varied considerably and the nuclei as well as the Golgi apparatus were positioned randomly. However, the cells of the top layer seemed to be morphologically polarized, with numerous microvilli on the apical membrane facing the apical media. Cells within the multilayer were either surrounded by neighbouring cells along their entire surface or they formed an intercellular lumen with at least one other cell. Although the former appeared to lack a distinct polarity, the latter clearly exhibited signs of polar organization, such as junctional complexes at the apex of lateral membranes and microvilli on the luminal membrane. Cells facing the free surface as well as participitating in the formation of the intercellular lumen, exhibited two distinct membranes with extensive microvilli, which were separated by junctional complexes from basolateral membranes (Fig. 2C). In contrast to the changes in cell adhesion observed in multilayers formed upon transformation (Schoenenberger et al., 1991), the cells of the hormonally induced multilayer were in close contact to each other and to the filter support.

Induction of multilayering and lumen formation by prostaglandin E_1

These morphological data indicate that one or more components of the serum-free, hormone-supplemented media resulted in the formation of MDCK multilayers. To identify the factor(s) we analyzed the effect of the omission of different hormones from the serum-free culture media. Since transferrin is essential for optimal growth of MDCK cells in serum-free media (Taub et al., 1979), all hormone-depletion experiments were carried out in the presence of transferrin. The growth of MDCK cells in serum-free media containing only transferrin (Fig. 3B) was different from that in media supplemented with all five factors (Fig. 3A). The depletion of insulin, PGE_1, hydrocortisone and triiodothyronine resulted in the formation of a continous monolayer. No multilayering or lumen formation could be observed and the morphology was similar to cells grown in media with 5% fetal calf serum (Fig. 2A). Interestingly, the same growth properties were also obtained when only PGE_1 was omitted from the hormone supplement (Fig. 3C). These findings suggest that PGE_1 is involved in cyst formation. In order to verify this observation further, we seeded MDCK cells in serum-free media supplemented with only PGE_1 and transferrin. Analysis by electron microscopy showed MDCK cell multilayers with intercellular lumina (Fig. 3D), with the same morphology as found for MDCK cells cultured in serum-free media containing all five factors (Fig. 2B). Higher-power electron micrographs of the cells grown in the presence of PGE_1 showed an intercellular lumen formed by two cells which were connected by junctional complexes resembling tight junctions (Fig. 4A,B). Again, for the cells of the top layer, microvilli could be detected on both the free cell surface and the membrane

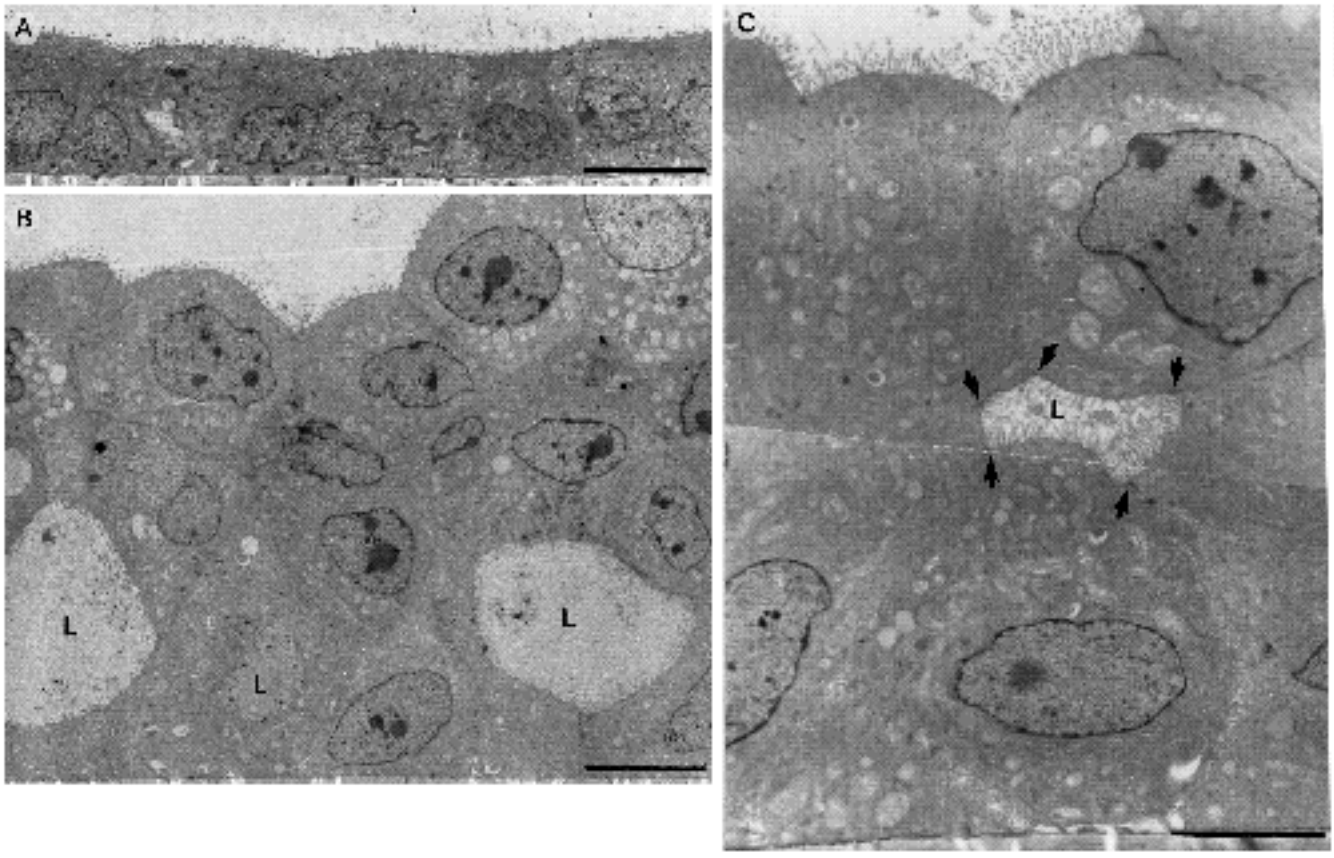

Fig. 2. Electron micrographs of MDCK cells grown in culture media containing FCS or serum-free media supplemented with hormones. Cells were seeded at high densities (2×10^6 cells/cm^2) on filters and grown for four days in culture media with (A) 5 % FCS or (B) serum-free media supplemented with insulin, transferrin, PGE_1, hydrocortisone and triiodothyronine. Lumina (L) are present in B. (C) Higher-power electron micrograph of cells grown under conditions as in B. The junctional complexes between adjacent cells forming a lumen (L) are indicated by arrows. Bars: A,B, 10 µm; C, 5 µm.

facing the lumen. In addition, the membrane domains were separated by junctional complexes.

Mechanism of action of prostaglandin E_1

Because PGE_1 had been identified as the factor that specifically induces multilayering and lumen formation of MDCK cells, the mechanism by which PGE_1 effects these growth properties was of interest. PGE_1 has been shown to stimulate adenylate cyclase, leading to elevated cyclic AMP levels in MDCK cells (Rindler et al., 1979). Furthermore, it has been reported that dibutyryl cyclic AMP at 0.5 mM substitutes for the growth-enhancing effect of PGE_1 on MDCK cells in serum-free medium (Taub et al., 1979). The possibility that PGE_1 acts via an increase in the intracellular cyclic AMP level was therefore studied. When MDCK cells were plated using serum-free medium containing only 0.5 mM dibutyryl cyclic AMP and transferrin (Fig. 3E), we observed morphological characteristics identical to those caused by PGE_1 (Fig. 3D). The degradation product of dibutyryl cyclic AMP, sodium butyrate, had no such effect (data not shown). In addition, another factor that affects the intracellular cyclic AMP level was studied. Isobutyl methylxanthine (IMBX), which is a potent inhibitor of phosphodiesterase, increases the cyclic AMP concentration in MDCK cells (Rindler et al., 1979). In the presence of 0.5 mM IMBX the formation of multilayers and intercellular lumina could be observed (Fig. 3F). The concentrations of PGE_1, dibutyryl cyclic AMP and IMBX which we used in these experiments resulted in a maximal stimulation of multilayer formation. The extent and degree of multilayering was similar for all three factors. Lower concentrations of PGE_1 ($\leq$ 12.5 ng/ml), dibutyryl cyclic AMP ($\leq$ 0.25 mM) and IMBX ($\leq$ 0.1 mM) reduced the abundance and stability of multilayers.

These findings suggest that PGE_1 induces drastic morphological changes in MDCK cells, which lead to the formation of extensive multilayers with abundant lumina. PGE_1 appears to act via elevation of the intracellular cAMP level, since dibutyryl cAMP and the phosphodiesterase inhibitor IMBX can substitute for the prostaglandin.

Cell surface polarity in MDCK cell multilayers induced by prostaglandin E_1

The presence of extensive microvilli at the free cell surfaces facing the medium and the lumen, with junctional complexes bordering these domains, suggested a polarized cell organization. We therefore analyzed the distribution of apical and basolateral cell surface markers by indirect immunofluorescence. Cryosections (8-10 µm) were stained with mAbs to a 58 kDa protein specific for the basolateral

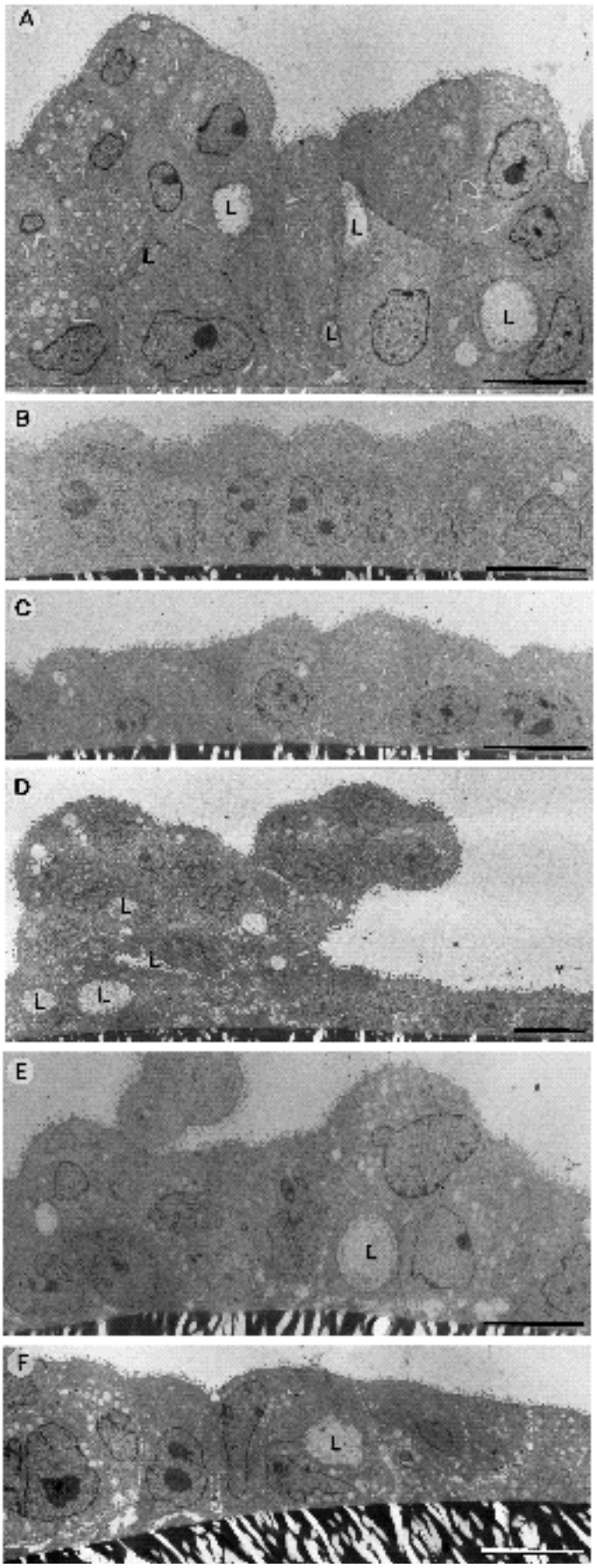

Fig. 3. Ultrastructure of MDCK cells grown in the presence or absence of multilayer-inducing factors. MDCK cells were cultured in serum-free medium supplemented with different factors: (A) insulin, transferrin, PGE_1, hydrocortisone and triiodothyronine. (B) Transferrin. (C) Insulin, transferrin, hydrocortisone and triiodothyronine. (D) Transferrin and PGE_1. (E) Transferrin and dibutyryl cyclic AMP. (F) Transferrin and isobutyl methylxanthine. Lumina (L) are present in A, D, E, F. Bar, 10 μm.

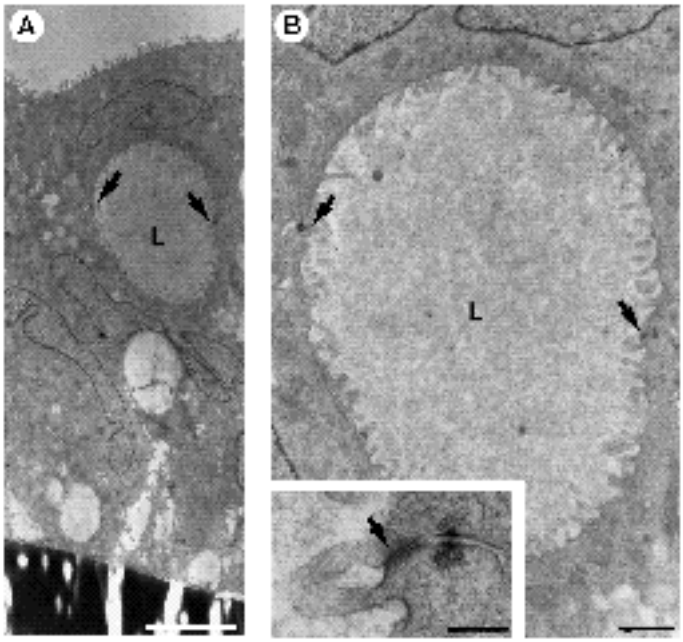

Fig. 4. Electron micrographs of MDCK cells grown in the presence of PGE_1 and transferrin. (A) In the upper portion of the multilayer a lumen (L) is visible, which is formed by two cells connected by junctional complexes (arrows). (B) Higher magnification of A. The inset shows the right junctional complex depicted in A and B, which resembles a tight junction. Bars: A, 5 μm; B, 1 μm; inset in B, 250 nm.

membrane (Balcarova-Ständer et al., 1984). In MDCK cells grown as a monolayer in serum-free media supplemented only with transferrin, labeling was detected exclusively at the basolateral surface (Fig. 5A). In multilayers induced by PGE_1 the 58 kDa protein was also excluded from the free cell surface of the top layer (Fig. 5B). However, for the cells within the multilayer staining appeared over the entire cell surface, suggesting the absence of cell polarity or at least of a defined apical domain. Consequently, we also analyzed the localization of an apical cell surface marker by incubating cryosections with mAbs to a 114 kDa apical membrane protein (Balcarova-Ständer et al., 1984). In control monolayers the free cell surface was labeled but no staining could be detected at the basolateral membranes (Fig. 6A). The same apical distribution of the 114 kDa protein was found in the cells of the top layer of PGE_1-induced multilayers where staining was detected only at the free surface (Fig. 6B). Within the multilayer no staining was observed except for the surface of round structures, which we believe to be the lumina of cysts.

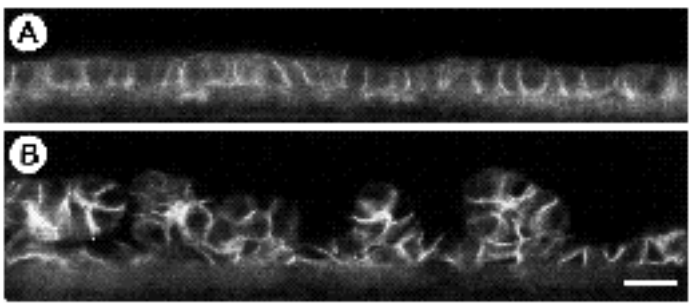

Fig. 5. Distribution of the basolateral 58 kDa membrane protein in control and MDCK cells grown in the presence of PGE_1. Cryosections about 8 µm thick of cells grown in serum-free media supplemented with (A) transferrin or (B) transferrin and PGE_1 were incubated with mAbs to a 58 kDa protein specific for the basolateral membrane, followed by FITC-coupled anti-mouse secondary antibodies. Bar, 20 µm.

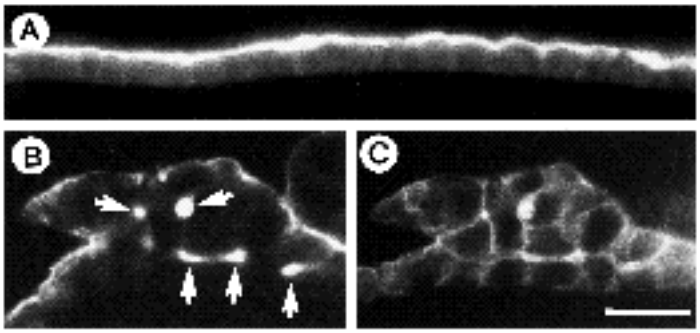

Fig. 6. Immunofluorescence staining of MDCK cells grown as a monolayer (control) or in serum-free medium plus PGE_1. MDCK cells grown in serum-free medium supplemented with (A) transferrin or (B) transferrin and PGE_1 were stained for the apical 114 kDa surface marker. (C) The same section as in B was stained a second time with mAb 58 to label the basolateral membranes for demonstration of the relative position of cysts (arrows in B) marked by mAb 114 in B. Bar, 20 µm.

In order to gain further evidence for the intercellular localization of these labeled putative lumina, we performed a second immunofluorescence staining of the same section (Fig. 6C). As we expected, further incubation with mAbs to the basolateral 58 kDa protein resulted in additional staining of formerly unlabeled basolateral membranes. So, the visualized relative position of the initialy stained membranes supported our idea that we had labeled intercellular lumina with the mAb to the apical marker protein.

While the presence of microvilli and of an apical marker protein indicated that the luminal sides of the cysts were organized like an apical membrane domain, we still had to examine whether the basolateral 58 kDa protein was excluded from the luminal membrane. Direct evidence came from pre-embedding/peroxidase staining of MDCK cells grown in the presence of PGE_1 and transferrin (Fig. 7). The mAb to the apical 114 kDa protein labeled both the free cell surfaces facing the culture medium and, if accessible, the luminal membrane of the cyst (Fig. 7A). In contrast, using mAbs to the 58 kDa protein, the basolateral marker was restricted to areas of cell-filter and cell-cell contact. No staining could be detected at the luminal surface (Fig. 7B).

In summary, these immunocytochemical data indicate that in MDCK cell multilayers induced by PGE_1 the uppermost layer displays the polarized organization of a monolayer with an apical and a basolateral membrane domain separated by junctional complexes. Interestingly, also, the cells within the multilayer show membrane polarization, since the basolateral marker is exclusively localized to areas of cell contact, which is for most of these cells the entire cell surface; whereas the apical marker is only present on the luminal membrane of the intercellular lumen.

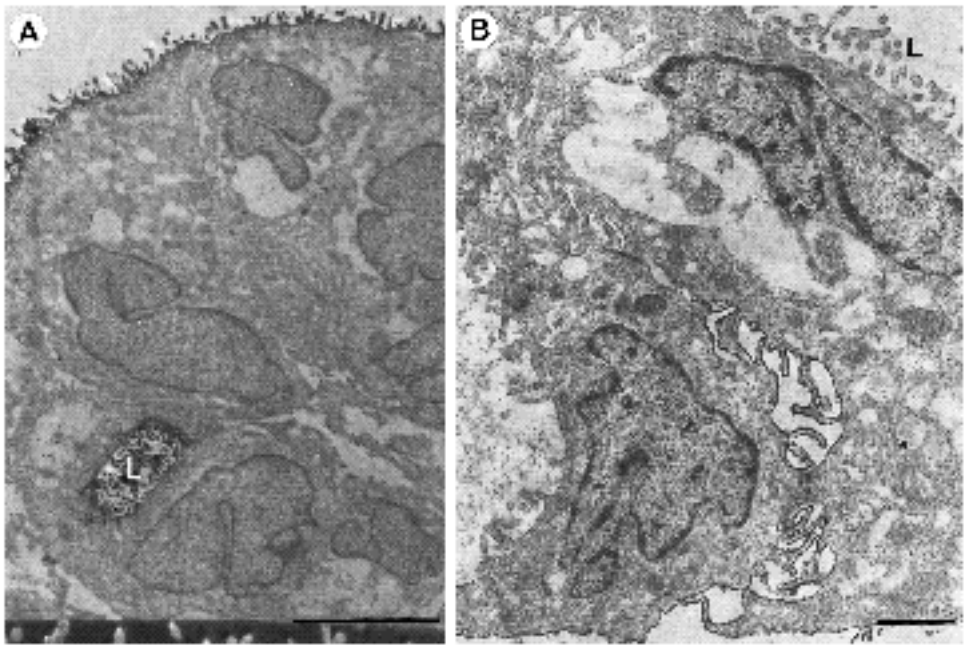

Fig. 7. Pre-embedding-immunoperoxidase staining of MDCK cells grown in serum-free medium plus PGE_1. MDCK cells grown in the presence of transferrin and PGE_1 were incubated with mAbs to the apical 114 kDa protein (A) or to the basolateral 58 kDa protein (B) followed by peroxidase-conjugated secondary antibodies. Lumina (L). Bars: A, 5 µm; B, 1 µm.

DISCUSSION

In the present study we analyzed the role of hormone-supplemented, serum-free medium on the morphology and cell surface polarity of the epithelial cell line MDCK. First, MDCK cells form multilayers with intercellular lumina when seeded at high densities in serum-free medium supplemented with insulin, transferrin, prostaglandin E_1, hydrocortisone and triiodothyronine. In contrast, when grown in medium containing FCS, independent of the plating density a continous monolayer is formed. Second, the presence of PGE_1 alone is sufficient to induce multilayer and lumen formation. Dibutyryl cyclic AMP, as well as the inhibitor of the phosphodiesterase IMBX, effectively substitutes for PGE_1 to permit the development of multicellular epithelial structures. Lastly, the multilayer exhibits a polarized phenotype with extensive microvilli on the free surface and the luminal membrane. Analysis of the distribution of membrane marker proteins demonstrates that apical domains are formed on the free cell surfaces while basolateral domains form at sites of cell contact. If more than one domain is present within a cell, the domains are separated by junctional complexes.

The defined medium supplemented with hormones and growth factors was originally designed for the long-term growth of MDCK cells (Taub et al., 1979). Omission of either PGE_1 or transferrin has been shown to be more deleterious to cell growth than omission of any other component. The action of PGE_1 on MDCK cells seems to be mediated by cyclic AMP, since dibutyryl cyclic AMP could substitute for the growth-stimulating effect of PGE_1. In addition, it has been observed that PGE_1 stimulates adenylate cyclase and leads to elevated cyclic AMP levels in MDCK cells (Rindler et al., 1979). The finding that PGE_1 acts via elevation of the intracellular cyclic AMP level is consistent with our results, since, in the formation of multicellular cysts, PGE_1 can be substituted by dibutyryl cyclic AMP or the phosphodiesterase inhibitor IMBX.

The unusual phenotype of multilayered epithelial cells tempted us to analyze the cell polarity at different positions of the multilayer. Until this report nontransformed MDCK cells have been described as forming only monolayered epithelial cysts, mainly when grown in collagen gel matrix (Hall et al., 1982; Karst and Merker, 1988; Mangoo-Karim et al., 1989). Wang et al. (1990a,b) have analyzed the generation of cell polarity in these monolayered MDCK cysts developed in collagen gel or in suspension culture. They found that the orientation of cell polarity depends upon whether cysts are formed in a collagen gel or in suspension culture. In collagen gels, the apical membrane with microvilli faces the central lumen, whereas in cells grown in suspension culture it faces the outside surface of cysts. In contrast, upon PGE_1-induced multilayer formation we detect microvilli and an apical cell surface marker on both the free cell surface of top layer cells and the luminal membrane of cysts. The basolateral 58 kDa marker is excluded from this domain and is restricted to areas of cell contact. The presence of both orientations of cell polarity within the MDCK cell multilayer, as they can be observed in cysts formed in suspension and collagen gel, is however consistent with the findings of Wang et al., who proposed that the orientation of cell polarity depends upon the localization of the basal lamina. The accumulation of secreted basal lamina proteins (Caplan et al., 1987) between the cells could explain why in PGE_1-induced multilayers the free cell surfaces facing the medium and the intercellular lumen are organized as apical membranes, whereas the areas of cell-cell and cell-substratum contact form the basolateral domains.

To our knowledge MDCK cell multilayers similar to those described here have been observed only when MDCK cells were transformed with the v-K-*ras* oncogene (Schoenenberger et al., 1991). However, multilayers were formed only when transformed cells were grown on permeable filter support, not on plastic substrata. Microvilli were less abundant and cell-substratum and cell-cell adhesion was decreased. In addition, the apical polarity was disrupted, since the apical 114 kDa membrane protein was randomly distributed on the cell surface, whereas the basolateral 58 kDa membrane protein remained exclusively localized to areas of cell contact. In contrast to the reduced cell adhesion of transformed MDCK cells (Warren and Nelson, 1987; Behrens et al., 1989; Schoenenberger et al., 1991), we find the cells of the multilayer closely attached to each other and to the filter support. Significantly, the cells fully retain their potential of apical and basolateral polarization. Apical membrane domains are formed at the free surfaces of the uppermost layer and the intercellular lumen. Areas of cell contact exhibit a basolateral polarity. No intermixing of apical and basolateral membrane markers is found, even for cells which are surrounded by neighbouring cells along their entire cell surface: these cells seem to develop only a basolateral domain, with the apical 114 kDa protein beeing strictly excluded from the contacting plasma membrane. However, it is most striking that cells participitating in the formation of the free cell surface as well as of the luminal membrane exhibit numerous microvilli on both of these membranes (Figs 2C, 4A,B). Furthermore, for these cells four junctional complexes resembling tight junctions can be detected, one at either end of each contact zone between adjacent cells. Considering extensive microvilli as a structural apical marker which clearly colocalizes with the apical 114 kDa marker, these findings could indicate the development of two apical domains within these cells. This has not been observed before in MDCK cells.

Our results suggest that cAMP influences the cell adhesion properties of MDCK cells. When grown at high density in the presence of PGE_1 with cells lying one upon the other, the increased cell adhesion could allow all cells to adhere. In the absence of PGE_1 the excess cells fall off and a regular monolayer is formed. This is consistent with the finding that an antibody against the cell adhesion molecule uvomorulin failed to dissociate a MDCK cell monolayer when the cellular cAMP level was raised (Behrens et al., 1985). Furthermore, PGE_2 was shown to increase the plating efficiency of rabbit tracheal epithelial cells, since in the presence of PGE_2 more cells attached to the collagen substratum in the first hours after plating (Zhou et al., 1990).

It is tempting to speculate that cAMP could act via an interaction with the cytoskeleton, since cell-cell adhesion molecules such as uvomorulin have been reported to be linked to components of the membrane-associated actin

cytoskeleton (Hirano et al., 1987; Ozawa et al., 1989; Nelson et al., 1990). Moreover, an elevation of the intracellular cAMP level in MDCK cells produced a rearrangement of actin bundles, but had no effect on the organization of microtubules (Mills and Lubin, 1986). One possibility is that cAMP could act through cAMP-dependent protein kinase (A-kinase) by phosphorylation of proteins associated with the actin cytoskeleton. Further experiments will therefore focus on the mechanism of action of cAMP in the formation of MDCK cell multilayers.

We are grateful to Kai Simons (EMBL, Heidelberg) for kindly providing monoclonal antibodies against the 114 kDa and the 58 kDa membrane proteins. In addition, we thank Marino Zerial and Mark Stapleton (EMBL, Heidelberg) for critical reading of the manuscript.

A large part of this research was done at the Institute of Biochemistry, University of Frankfurt/Main, Germany. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 169) and the Fond der Chemischen Industrie in cooperation with the Bundesministerium für Forschung und Technologie.

REFERENCES

Balcarova-Ständer, J., Pfeiffer, S. E., Fuller, S. D. and Simons, K. (1984). Development of cell surface polarity in the epithelial Madin-Darby canine kidney (MDCK) cell line. *EMBO J.* **3**, 2687-2694.

Behrens, J., Birchmeier, W., Goodman, S. L. and Imhof, B. A. (1985). 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. *J. Cell Biol.* **101**, 1307-1315.

Behrens, J., Mareel, M. M., Van Roy, F. M. and Birchmeier, W. (1989). Dissecting tumor cell invasion: epithelial cells acquire invasive properties after the loss of uvomorulin-mediated cell-cell adhesion. *J. Cell Biol.* **108**, 2435-2447.

Caplan, M. J., Stow, J. L., Newman, A. P., Madri, J., Anderson, H. C., Farquhar, M. G., Palade, G. E. and Jamieson, J. D. (1987). Dependence on pH of polarized sorting of secreted proteins. *Nature* **329**, 632-635.

Graham, R. C. and Karnovsky, M. J. (1966). The early stages of adsorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: Ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14**, 291-302.

Gumbiner, B. (1987). Structure, biochemistry, and assembly of epithelial tight junctions. *Amer. J. Physiol.* **5922**, C749-758.

Hall, H. G., Farson, D. A. and Bissell, M. J. (1982). Lumen formation by epithelial cell lines in response to collagen overlay: a morphogenetic model in culture. *Proc. Nat. Acad. Sci. USA* **79**, 4672-4676.

Hirano, S., Nose, A., Hatta, K., Kawakami, A. and Takeichi, M. (1987). Calcium-dependent cell-cell adhesion molecules (cadherins): subclass specificities and possible involvement of actin bundles. *J. Cell Biol.* **105**, 2501-2510.

Karst, W. and Merker, H.-J. (1988). The differentiation behaviour of MDCK cells grown on matrix components and in collagen gels. *Cell Differ.* **22**, 211-224.

Leighton, J., Estes, L. W., Mansukhani, S. and Brada, Z. (1970). A cell line derived from normal dog kidney (MDCK) exhibiting qualities of papillary adenocarcinoma and of renal tubular epithelium. *Cancer* **26**, 1022-1028.

Mangoo-Karim, R., Uchic, M., Lechene, C. and Grantham, J. J. (1989). Renal epithelial cyst formation and enlargement in vitro: dependence on cAMP. *Proc. Nat. Acad. Sci. USA* **86**, 6007-6011.

McLean, I. W. and Nakane, P. K. (1974). Periodate-lysine-paraformaldehyde fixative. A new fixative for immunoelectron microscopy. *J. Histochem. Cytochem.* **22**, 1077-1083.

McRoberts, J. A., Taub, M. and Saier, M. H., Jr (1981). The Madin-Darby canine kidney (MDCK) cell line. In *Functionally Differentiated Cell Lines* (ed. G. Sato), pp. 117-139. A. R. Liss, Inc., New York.

Mills, J. W. and Lubin, M. (1986). Effect of adenosine 3′,5′-cyclic monophosphate on volume and cytoskeleton of MDCK cells. *Amer. J. Physiol.* **250**, C319-C324.

Montesano, R., Matsumoto, K., Nakamura, T. and Orci, L. (1991a). Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. *Cell* **67**, 901-908.

Montesano, R., Schaller, G. and Orci, L. (1991b). Induction of epithelial tubular morphogenesis in vitro by fibroblast-derived soluble factors. *Cell* **66**, 697-711.

Nelson, W. J., Shore, E. M., Wang, A. Z. and Hammerton, R. W. (1990). Identification of a membrane-cytoskeletal complex containing the cell adhesion molecule uvomorulin (E-cadherin), ankyrin, and fodrin in Madin-Darby canine kidney epithelial cells. *J. Cell Biol.* **110**, 349-357.

Ojakian, G. K. and Schwimmer, R. (1988). The polarized distribution of an apical cell surface glycoprotein is maintained by interactions with the cytoskeleton of Madin-Darby canine kidney cells. *J. Cell Biol.* **107**, 2377-2387.

Ozawa, M., Baribault, H. and Kemler, R. (1989). The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. *EMBO J.* **8**, 1711-1717.

Rindler, M. J., Chuman, L. M., Shaffer, L. and Saier, M. H., Jr (1979). Retention of differentiated properties in an established dog kidney epithelial cell line (MDCK). *J. Cell Biol.* **81**, 635-648.

Rodriguez-Boulan, E. and Nelson, W. J. (1989). Morphogenesis of the polarized epithelial cell phenotype. *Science* **245**, 718-725.

Schoenenberger, C.-A. and Matlin, K. S. (1991). Cell polarity and epithelial oncogenesis. *Trends Cell Biol.* **1**, 87-92.

Schoenenberger, C.-A., Zuk, A., Kendall, D. and Matlin, K. S. (1991). Multilayering and loss of apical polarity in MDCK cells transformed with viral K-ras. *J. Cell Biol.* **112**, 873-889.

Simons, K. and Fuller, S. D. (1985). Cell surface polarity in epithelia. *Annu. Rev. Cell Biol.* **1**, 243-288.

Spurr, A. R. (1969). A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* **26**, 31-43.

Taub, M., Chuman, L., Saier, M. H., Jr and Sato, G. (1979). Growth of Madin-Darby canine kidney epithelial cell (MDCK) line in hormone-supplemented, serum-free medium. *Proc. Nat. Acad. Sci. USA* **76**, 3338-3342.

van Meer, G. and Simons, K. (1986). The function of tight junctions in maintaining differences in lipid composition between the apical and basolateral cell surface domains of MDCK cells. *EMBO J.* **5**, 1455-1464.

Vega-Salas, D. E., Salas, P. J. I., Gundersen, D. and Rodriguez-Boulan, E. (1987). Formation of the apical pole of epithelial (Madin-Darby canine kidney) cells: polarity of an apical protein is independent of tight junctions while segregation of a basolateral marker requires cell-cell interactions. *J. Cell Biol.* **104**, 905-916.

Wang, A. Z., Ojakian, G. K. and Nelson, W. J. (1990a). Steps in the morphogenesis of a polarized epithelium. I. Uncoupling the roles of cell-cell and cell-substratum contact in establishing plasma membrane polarity in multicellular epithelial (MDCK) cysts. *J. Cell Sci.* **95**, 137-151.

Wang, A. Z., Ojakian, G. K. and Nelson, W. J. (1990b). Steps in the morphogenesis of a polarized epithelium. II. Disassembly and assembly of plasma membrane domains during reversal of epithelial cell polarity in multicellular epithelial (MDCK) cysts. *J. Cell Sci.* **95**, 153-165.

Warren, S. L. and Nelson, W. J. (1987). Nonmitogenic morphoregulatory action of $pp60^{v\text{-}src}$ on multicellular epithelial structures. *Mol. Cell. Biol.* **7**, 1326-1337.

Zhou, S., Mardini, I., Hilfer, R., Blank, K. and Kelsen, S. G. (1990). Effect of PGE_2 and PGF_2 on rabbit tracheal epithelial cell attachment and proliferation. *J. Cell Biol.* **111**, 441a (Abstract).

(Received 27 July 1992 - Accepted, in revised form, 3 November 1992)