Cytoskeletal Control of Centrioles Movement During The Establishment of Polarity in Madin-Darby Canine Kidney Cells

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Abstract. The two centrioles that are localized close to each other and to the nucleus in single Madin-Darby Canine kidney cells (MDCK) move apart by distances as large as 13 μm after the establishment of extensive cellular junctions. Microfilaments, and possibly microtubules appear to be responsible for this separation. In fully polarized cells, the centrioles are localized just beneath the apical membrane. After disruption of intercellular junctions in low calcium medium, the centrioles move back towards the cell center. This process requires intact microtubules but happens even in the absence of microfilaments. These results indicate that the position of centrioles is determined by opposing forces produced by microtubules and microfilaments and suggest that the balance between these forces is modulated by the assembly of cellular junctions. Centriole separation appears to be an early event in the process that precedes their final positioning in the apical-most region of the polarized cell.

In vivo, epithelia perform a boundary function between the external and internal milieu. This function requires a specific cellular and intercellular organization that is expressed in the polarized state of epithelial cells. The plasma membrane of these cells is divided into an apical domain exposed to the external milieu and a basolateral domain, surrounded by interstitial fluid. The two domains are separated by tight junctions that prevent free exchanges between the two fluids as well as intermixing of basolateral and apical proteins in the plane of the plasma membrane (Madara, 1988; Simons, 1989). The epithelial sheet is further stabilized by adherens junctions and desmosomes that interact with actin filaments and the cytokeratin network, respectively (Geiger et al., 1985; Steinberg et al., 1987; Franke et al., 1987).

The polarized cell state found in epithelia is reproduced in vitro in some tissue culture systems. The Madin-Darby canine kidney (MDCK) cell line represents such an example and has been widely used to address questions related to epithelial cell polarity (Simons and Fuller, 1985). This cell line is attractive to study the generation and maintenance of epithelial cell polarity because polarity can be disrupted by trypsinization during passaging and is progressively reestablished after the establishment of cell contacts (Balcarova-Stander et al., 1984). Moreover, after exposure of the cells to low calcium medium, the various junctions are destabilized and cell polarity is lost. When the cells are transferred back to normal tissue culture medium, the junctions reform and cell polarity is reestablished (Hoi-Sang et al., 1979; Martinez-Palomo et al., 1980; Meza et al., 1980; for review, see Geiger, 1985; Mattey and Garrod, 1986; Vega-Salas et al., 1987, 1988). Finally, antibodies directed against cell junction components interfere with the fence function and prevent the establishment of cell polarity (Imhof et al., 1983; Behrens et al., 1985; Gumbiner et al., 1986; Herzlinger and Ozakian, 1984). This provides a model to investigate what is the causal relationship between junction assembly, cytoskeleton reorganization, and epithelial cell polarization.

Although conflicting data have been reported concerning the involvement of microtubules and microfilaments in the generation and maintenance of epithelial cell polarity (Rindler et al., 1987; Salas et al., 1986), both theoretical considerations and structural observations support the idea that they play a fundamental role (Volberg et al., 1986; Green et al., 1987; Hirano, 1987). Both filament types are polar structures whose ends are not equivalent. The "plus" end of microtubules is more active than the "minus" end: it grows and shrinks faster, and more often, than the minus end (Mitchison and Kirschner, 1984; Kirschner and Mitchison, 1986; Horio and Hotani, 1986), while the "barbed" end of microfilaments grows faster than the "pointed" end (Bonder et al., 1983). Microtubules grow with the plus end away from the nucleating site and cytoplasmic motors that translocate organelles or beads along microtubules can "read" their polarity. For example, kinesin will move towards the plus end of microtubules, whereas MAP1c moves towards the minus end (Paschal and Vallee, 1987; Vale, 1987). Therefore, the location of a nucleating site in the cell determines the orientation of the microtubules in the cytoplasm and, as a consequence, the vectorial movement as well as the steady state position of organelles such as the Golgi apparatus, that slide along microtubules in one direction (for review, see Thyberg and

Moskalewski, 1985; Kreis et al., 1988). Actin microfilaments can also be oriented in the cell by having the barbed end bound to the junctional plaque for example (Begg et al., 1978). It follows that these polar filamentous systems can, in principle, determine the overall polarity of the cytoplasm in response to external signals (Bershadsky and Vasiliev, 1988).

The positioning of microtubule-nucleating sites is of particular interest in the study of the generation of polarity in MDCK cells since this can determine the orientation of microtubules and therefore the directional transport of specific vesicles as well as the location of the Golgi apparatus (Bacallao et al., 1989). As a consequence, this could affect the targeting of specific proteins to the apical and basolateral domains (Hoi-Sang et al., 1979; Simons and Fuller, 1985; Vega-Salas et al., 1987).

In the present work, we show that in isolated MDCK2 cells, the centrioles are located close to each other and to the nucleus, as in fibroblasts (Wheatley, 1982). After the establishment of cell contacts, the centrioles separate by a mechanism dependent on intact microfilaments. This seems to be the initial step in the process that leads to the final location of centrioles under the apical membrane of polarized cells. After the exposure of subconfluent cells to low calcium medium, intercellular junctions dissociate and the centrioles move back together in a microtubule-dependent way. These results suggest that in MDCK2 cells, the location of centrioles is modulated by the assembly of cellular junctions and determined by the conflicting action of microtubules and actin microfilaments.

Materials and Methods

Cells

MDCK epithelial cells strain II were grown in MEM with Eagle's salts supplemented with 10 mM Hepes (pH 7.3), 2 mM L-glutamine, 5% FCS, penicillin (110 U/ml), and streptomycin (100 μg/ml). The cells were seeded on glass coverslips and incubated in a humidified atmosphere, equilibrated with 5% CO_2 in air at 37°C.

Low Calcium Medium

Cells were seeded at a density that led to confluency after 3 d of culture. Subconfluent cells were used 32 h after seeding. The coverslips were washed once in PBS without Ca^{++} and once in low Ca^{++} medium containing EGTA. The low Ca^{++} medium was composed of MEM without calcium and supplemented with 10 mM Hepes, 2 mM L-glutamine, penicillin (110 U/ml), streptomycin (100 μg/ml), and 5% FCS previously dialyzed (24 h against NaCl 0.15 M; 24 h against NaCl 0.15 M, EGTA 0.2 mM, and 24 h against NaCl 0.15 M). To adjust the free calcium concentration in the culture to 2 μM, (EGTA-Ca^{++}) and EGTA solutions (200 mM each) were mixed in the ratio of 9:1 and diluted 100 times in the medium.

Drug Treatments

All experiments were carried out on subconfluent cells. In each case, the cells were washed once in PBS without Ca^{++} and once in the medium (MEM or Low Ca^{++} medium) containing the appropriate drug(s). Nocodazole (Sigma Chemical GmbH, Deisenhofen, FRG) was kept as a stock solution in DMSO at −20°C and diluted in the culture medium at 33 μm just before use. This concentration induced an almost complete depolymerization of microtubules in ~1 h. Cytochalasine D (Sigma Chemical Co., St. Louis, MO) was used at a final concentration of 1 μg/ml.

Antibodies

Rabbit antitubulin was a gift from Jan De Mey, the mouse monoclonal anti-uvomorulin was provided by K. Simons (Gumbiner and Simons, 1986), the monoclonal anticentrosome (CTR453) was obtained by immunization of mice with human centrosomes purified according to Bornens et al. (1987). It was a gift from M. Bornens. All secondary antibodies were "affinipure" antibodies purchased from Dianova GmBH (Hamburg, FRG).

Immunofluorescence

Double staining for tubulin and uvomorulin or tubulin and centrioles, was carried out on cells preextracted with Triton X-100 and fixed in methanol at −20°C as previously described (Bre et al., 1987). Tubulin and uvomorulin were labeled, using a mixture of rabbit antitubulin (1:100 dilution) and mouse antiuvomorulin (1:1,000). Secondary antibodies were Texas red-labeled goat anti-rabbit (1:100) and fluorescein-labeled sheep anti-mouse (1:50). Tubulin and centrosomes were labeled by the rabbit antitubulin (1:100) and the mouse anticentrosome (1:1,000). Secondary antibodies were fluorescein-labeled goat anti-rabbit (1:100) and Texas red-labeled rat anti-mouse (1:20). In all cases, the coverslips were washed three times in PBS, dipped quickly in ethanol and mounted in Mowiol.

Double staining for tubulin and actin, was carried out on cells fixed in glutaraldehyde as follows: after a brief rinse (2 s) in PBS at 37°C, the coverslips were incubated for 10 min at 37°C in 80 mM K-Pipes (pH 6.8), 5 mM EGTA, 1 mM $MgCl_2$, 0.5% Triton X-100, and 0.3% glutaraldehyde. The cells were rinsed briefly in PBS and incubated for 15 min at room temperature in $NaBH_4$ (1 mg/ml in PBS). This solution should be prepared just before use. It should produce bubbles. The cells were then washed once in PBS and two times for 5 min in PBS containing 0.1% Triton X-100. The tubulin was decorated with the rabbit antibody and after two washes in PBS containing 0.1% Triton X-100 (3 min each), rhodamine-labeled phalloidine (Sigma Chemical Co.) was added together with fluorescein-labeled goat anti-rabbit (1:100). After 10 min of incubation at room temperature, the coverslips were washed and mounted for observation.

Determination of Intercentriolar Distance and Data Analysis

The intercentriolar distance was measured on prints of cells stained with the monoclonal anticentrosome antibody. Images of the prints were displayed on a television monitor using a video camera (Lancaster, PA) (TC 10055/U). The distances were determined using an IBAS (Zeiss, Oberkochen, FRG) image processing system. Measurements were done for a minimum of 120 cells, and the data was arranged in 34 classes of 0.4 μm each. The frequency of each class is given in percentages of the total number of cells analyzed in a given experiment.

Electron Microscopy

Cells grown on plastic coverslips were briefly washed in PBS, preextracted with stabilizing medium and fixed with 0.3% glutaraldehyde in the same medium at 37°C for 10 min. Free aldehyde groups were blocked by sodium borohydride (1 mg/ml in PBS, pH 8) for 7 min. After washing in PBS, the coverslips were covered with a polyclonal rabbit antitubulin antibody for 30 min and washed 5 times (15 min each). The coverslips were then incubated for 30 min with gold-labeled protein A (8-nm size). After an overnight wash in PBS containing 0.1% Triton X-100, the cells were postfixed in 1% glutaraldehyde in 80 mM K-Pipes pH 6.8, 5 mM EGTA, and 1 mM $MgCl_2$. The specimens were then treated with 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.9) followed by 0.5% uranyl acetate in water and embedded in Epon. Serial thick (0.15–0.25 μm) and thin sections were cut and observed in an electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ).

Results

In MDCK Cells, Centrioles Separate after the Establishment of Cell Contacts

In isolated cells, centrioles were almost always located close to each other and to the nucleus as in fibroblasts (Fig. 1, *a–b*). Measurements carried out on 100 such cells showed that the intercentriolar distance was <2 μm in >80% of the cells. In subconfluent cells, the two centrioles were separated by a variable distance ranging from 0.4 to 13 μm (Figs. 1, *c–d*, and 5, MEM). In the electron microscope, they were

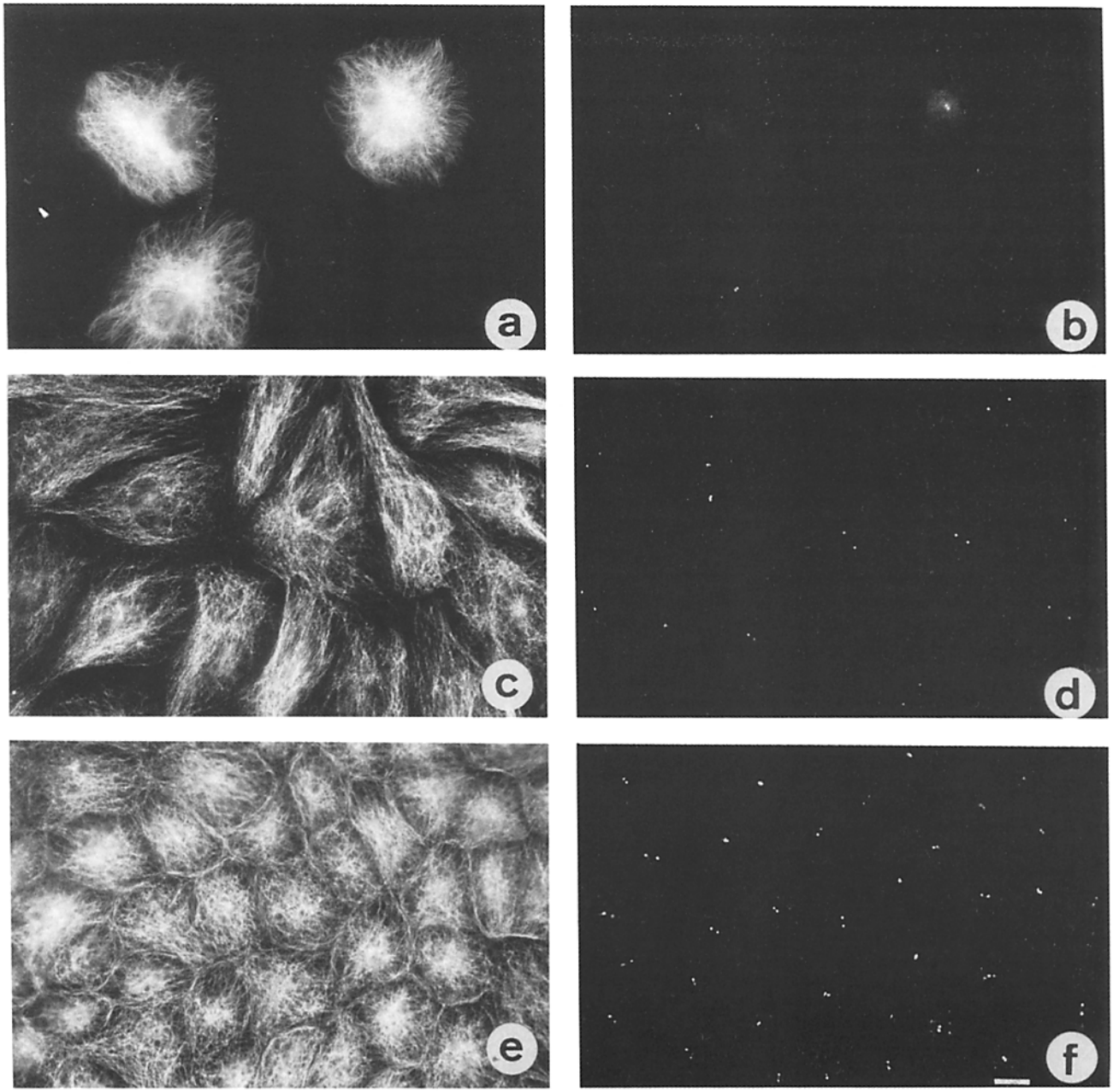

Figure 1. Centriole movement during the polarization of MDCK II cells. Cells were grown on glass coverslips, fixed with methanol for 5 min at −20°C, and processed for double immunofluorescence. Microtubule staining (*a*, *c*, and *e*), centriole staining (*b*, *d* and *f*). In isolated cells (*a* and *b*), centrioles are close to each other. They split in subconfluent cells (*c* and *d*) and move back together above the nucleus in polarized cells (*e* and *f*). Bar, 10 μm.

found anywhere in the cytoplasm, below, above, or on the side of the nucleus (Bré et al., 1987). Fig. 2 shows a stereo pair of one thick section chosen from a series of serial sections through an area of the cell in which only one centriole was present close to the nucleus. Microtubules were labeled by immunogold using a preembedding protocol. Although many microtubules were present in this section, only a few appeared to interact with the centriole. In fully confluent cells, the centrioles were closer to each other and always located well above the nucleus (Fig. 1 *f*). It was possible to locate the centrioles by focussing up and down through the cell, the nucleus being stained with a fluorescent dye. In the electron microscope, by serial sectioning parallel to the supporting coverslips, the centrioles were always found immediately below the apical plasma membrane (Fig. 3 *a*). Again, they did not nucleate many microtubules (Fig. 3 *b*).

The morphology of the microtubule network also changed dramatically during the establishment of polarity. In isolated cells, microtubules seemed to originate from a broad region containing the centrioles and located close to the nucleus (Fig. 1, *a–b*). In subconfluent cells, the network did not seem to originate from a specific area (Fig. 1 *c*), and finally it rearranged into a complex pattern in fully confluent cells (Fig. 1 *e*). A more detailed study of microtubule reorganization

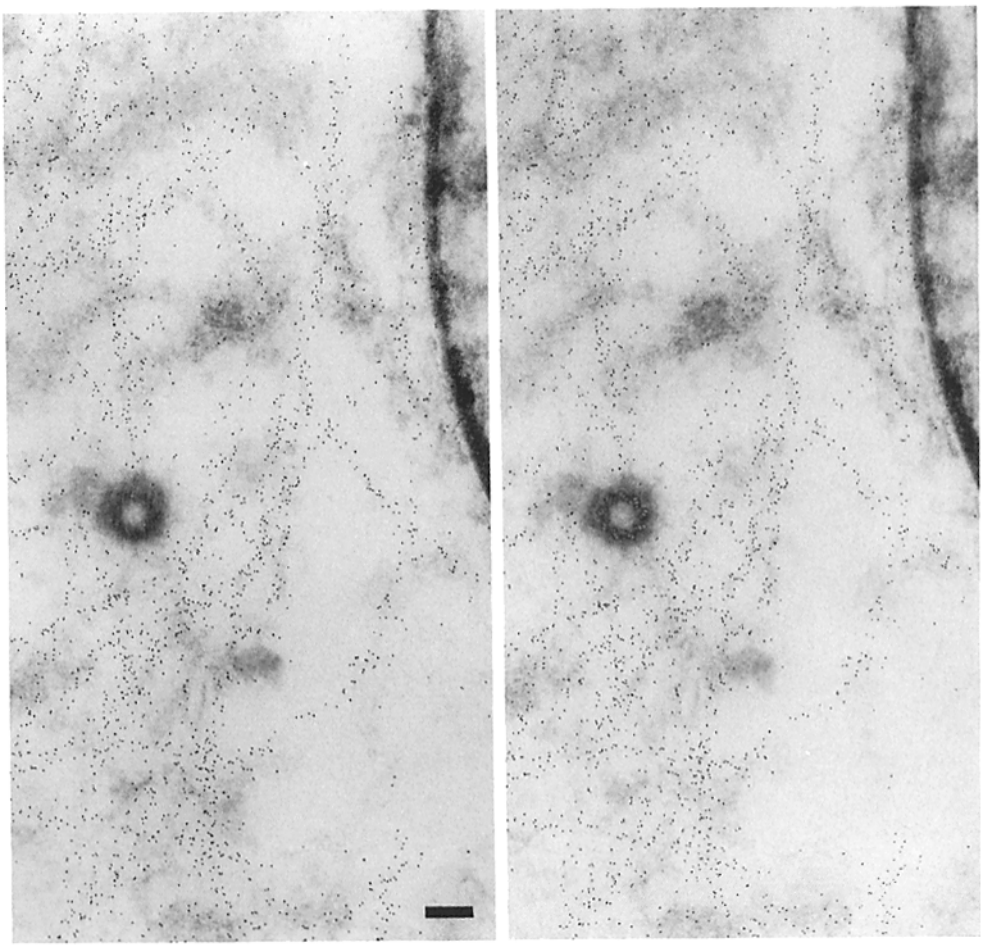

Figure 2. Localization of centrioles in subconfluent MDCK cells by electron microscopy. Cells were fixed and stained for microtubules by immunogold as described in materials and methods. The 2 centrioles were usually found far apart from each other and often in two entirely different regions of the cell. Here we show one single centriole next to the nucleus. It does not nucleate many microtubules. Magnification at 31,000. Bar, 200 nm.

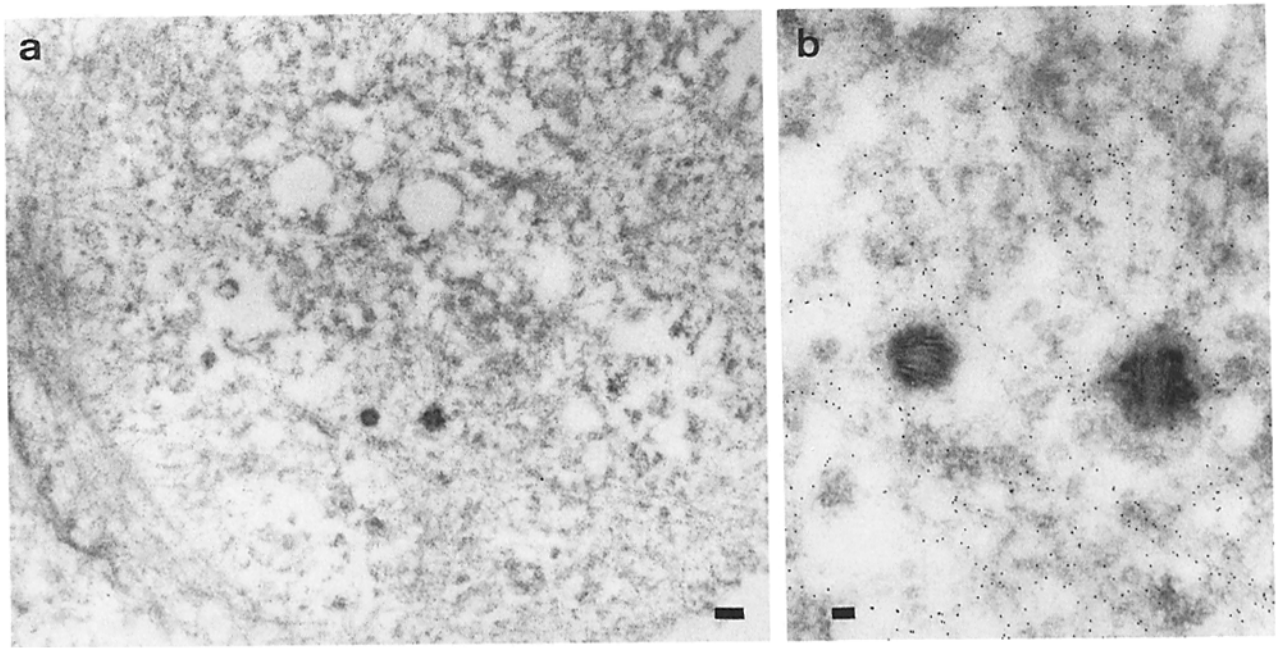

Figure 3. Localization of centrioles in polarized MDCK cells by electron microscopy. The two centrioles were always found close to each other just below the apical membrane. This was determined by observing serial sections cut perpendicular to the apicobasal axis of the cells. (*a*) 4,000×; (*b*) 16,000×. Bars, (*a*) 1 μm; (*b*) 200 nm.

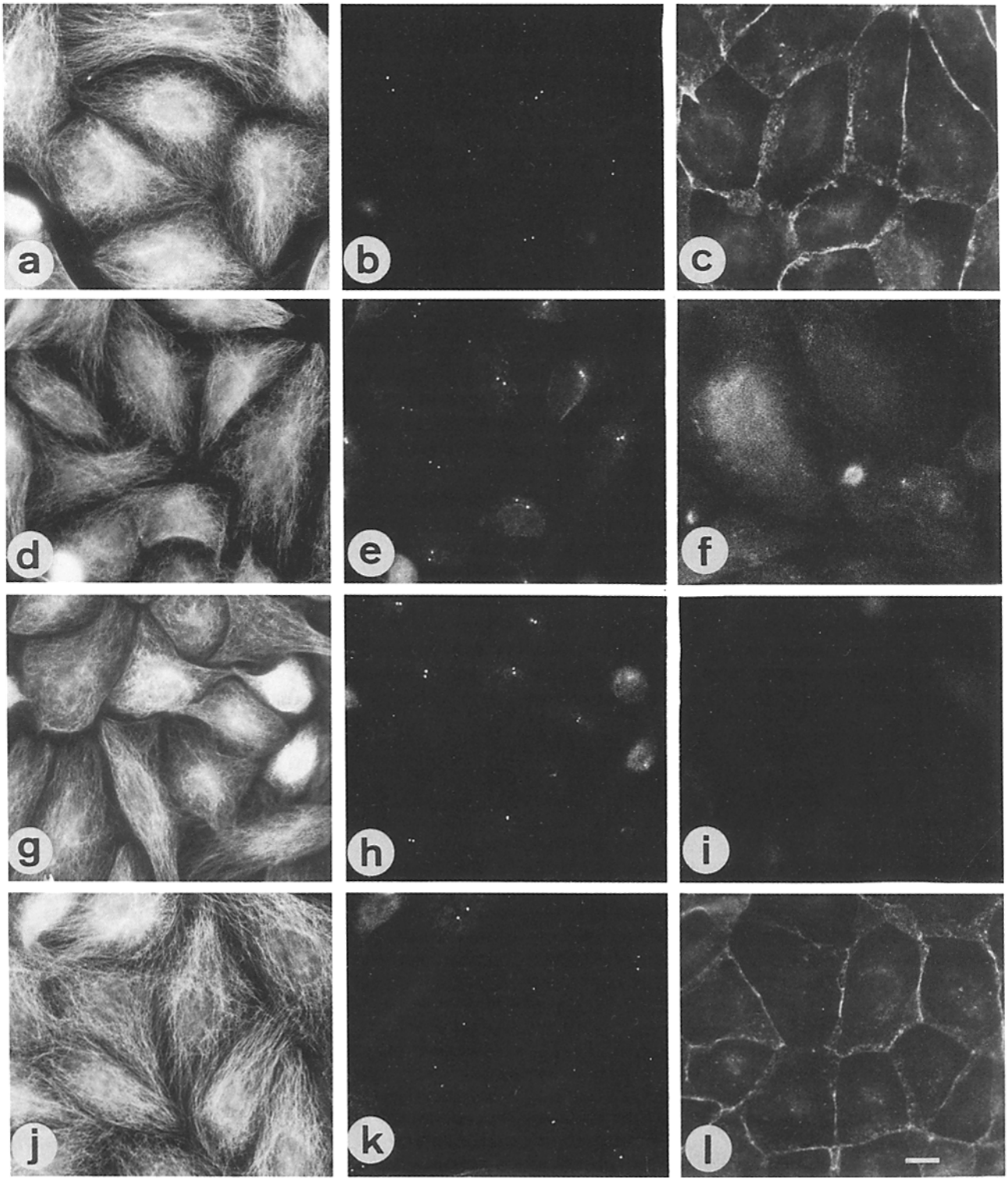

Figure 4. Centrioles move towards each other following junction disruption in low calcium medium. MDCK II cells were grown to subconfluency and fixed in methanol before (*a*, *b*, and *c*) or after incubation in low calcium medium for 30 min (*d*, *e*, and *f*) or 2 h (*g*, *h*, and *i*). After 2 h in low calcium medium, the cells were transferred back to normal medium and fixed 2.5 h later (*j*, *k*, and *l*). Cells were stained for microtubules (*left*), centrioles (*center*), and uvomorulin (*right*). Bar, 10 μm.

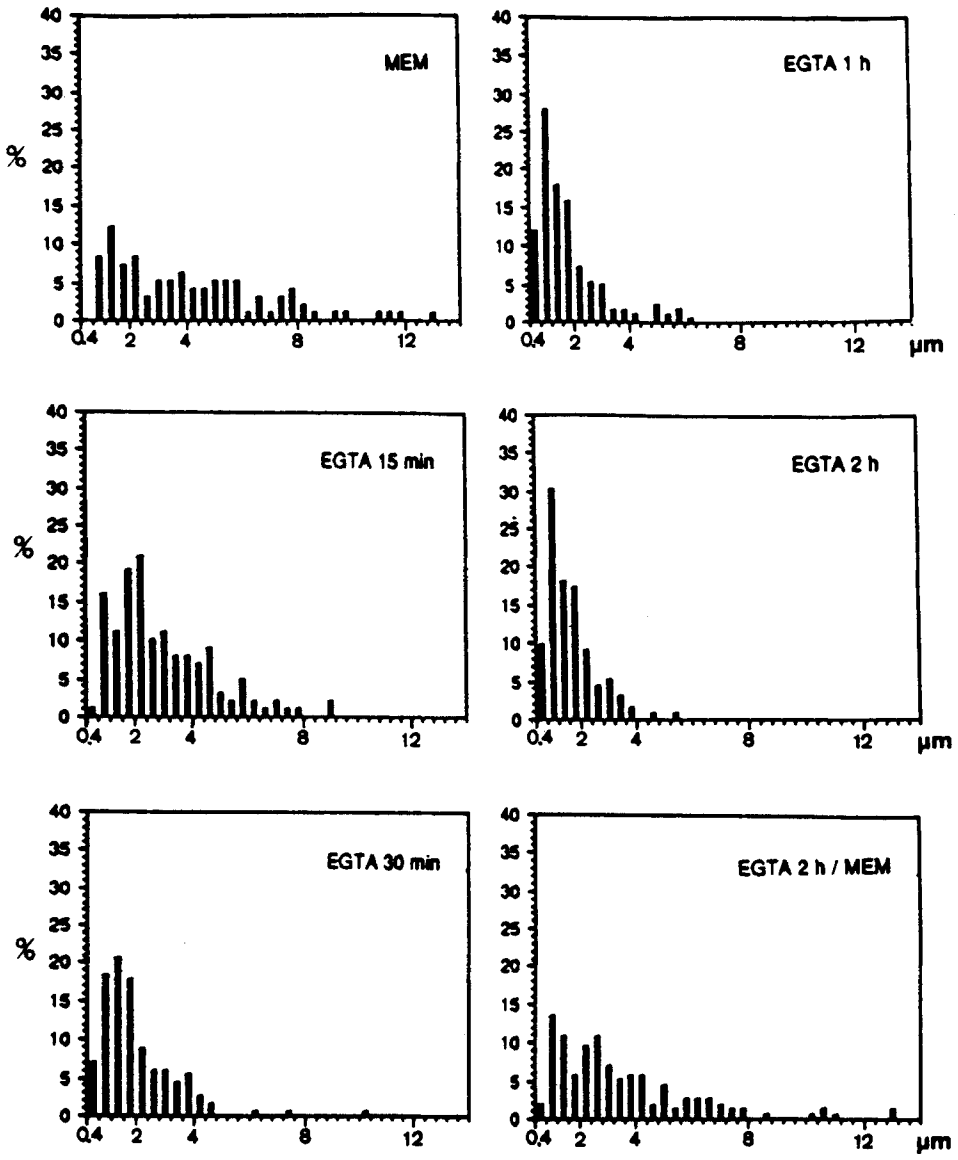

Figure 5. Quantification of intercentriolar distance in cells exposed to low calcium medium. The distance between centrioles was determined before (*MEM*), after various times of incubation in low calcium medium (EGTA), and after transfer to normal medium for 2.5 h (*EGTA 2 h/MEM*). The data is ordered in 34 classes, each class corresponding to an interval of 0.4 μm (*abscissa*). This figure shows the percentage of cells (from 0 to 40%, *ordinate*) containing centrioles separated by 0.4–0.8 μm (first class on the left) to 13.6–14 μm (*last class on the right*).

during the establishment of polarity in cells grown on filter supports is reported in a separate paper (Bacallao et al., 1989).

Effect of Low Calcium Medium on Cellular Junctions and Centrioles Separation

The observations described above suggested that centrioles separated in response to the establishment of cellular junctions. This was further studied by exposing subconfluent cells (Fig. 1, *c–d*) to low calcium medium, a treatment known to disrupt cellular junctions. All subsequent experiments were carried out under standard conditions, the cells being seeded at a given density, and fixed 32 h later. The kinetics of adherens junction disruption after exposure of the cells to low calcium medium was monitored by the disappearance of uvomorulin from the cell periphery with an antiuvomorulin antibody (Gumbiner and Simons, 1986; Gumbiner et al., 1988). Subconfluent cells were surrounded by a continuous rim of uvomorulin (Fig. 4 *c*) that disappeared 30 min after exposure of the cells to low calcium medium (Fig. 4, *f* and *i*). This timing was in good agreement with previous studies (Kartenbech et al., 1982; for review, see Edelman, 1985; Volberg et al., 1986; Mattey et al., 1986). The overall microtubule pattern showed some rearrangement during junctions disruption (Fig. 4, *a*, *d*, and *g*). The centrioles moved toward each other after transfer of the cells to low calcium medium with a kinetics that precisely followed disruption of the junctions (Fig. 4, *b–h*). In subconfluent cells, the intercentriolar distance was highly variable, ranging from 0.4 μm to 13 μm. Only 27% of the cells had centrioles separated by <2 μm (Fig. 5). We consider arbitrarily that centrioles separated by <2 μm are close to each other. After transfer to low calcium medium, the frequency of cells with centrioles separated by <2 μm increased progressively and reached 75% at 2 h (Fig. 5). This is close to the value found in populations of isolated cells maintained in normal medium (85%). When the subconfluent cells were transferred back to normal medium, the centrioles split again but only after extensive junction reassembly (Figs. 4 *k* and 5, *EGTA 2 h/MEM*). 2.5 h after transfer to normal culture medium, a normal cell shape was reestab-

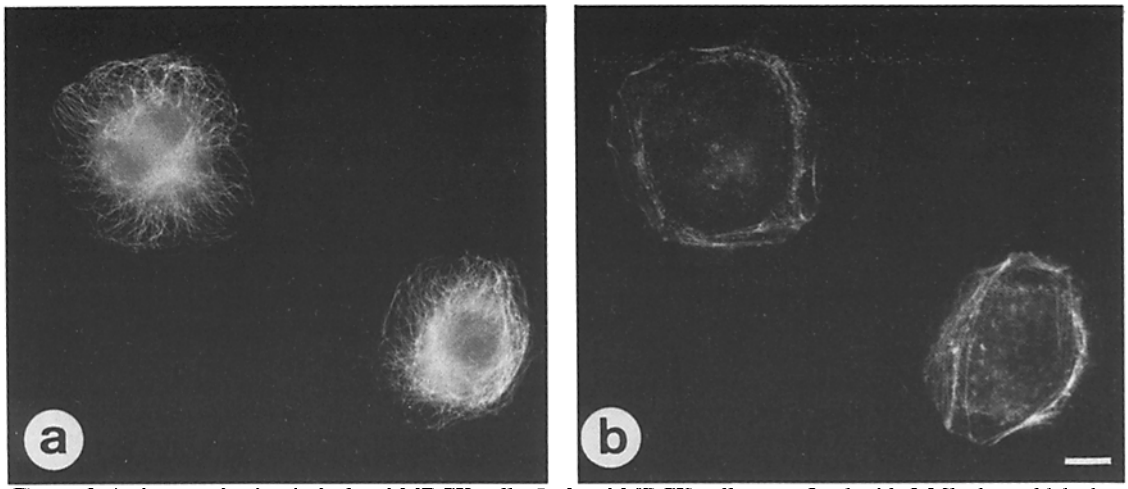

Figure 6. Actin organization in isolated MDCK cells. Isolated MDCK cells were fixed with 0.3% glutaraldehyde and double stained for tubulin (*a*) and for actin (*b*). Bar, 10 μm.

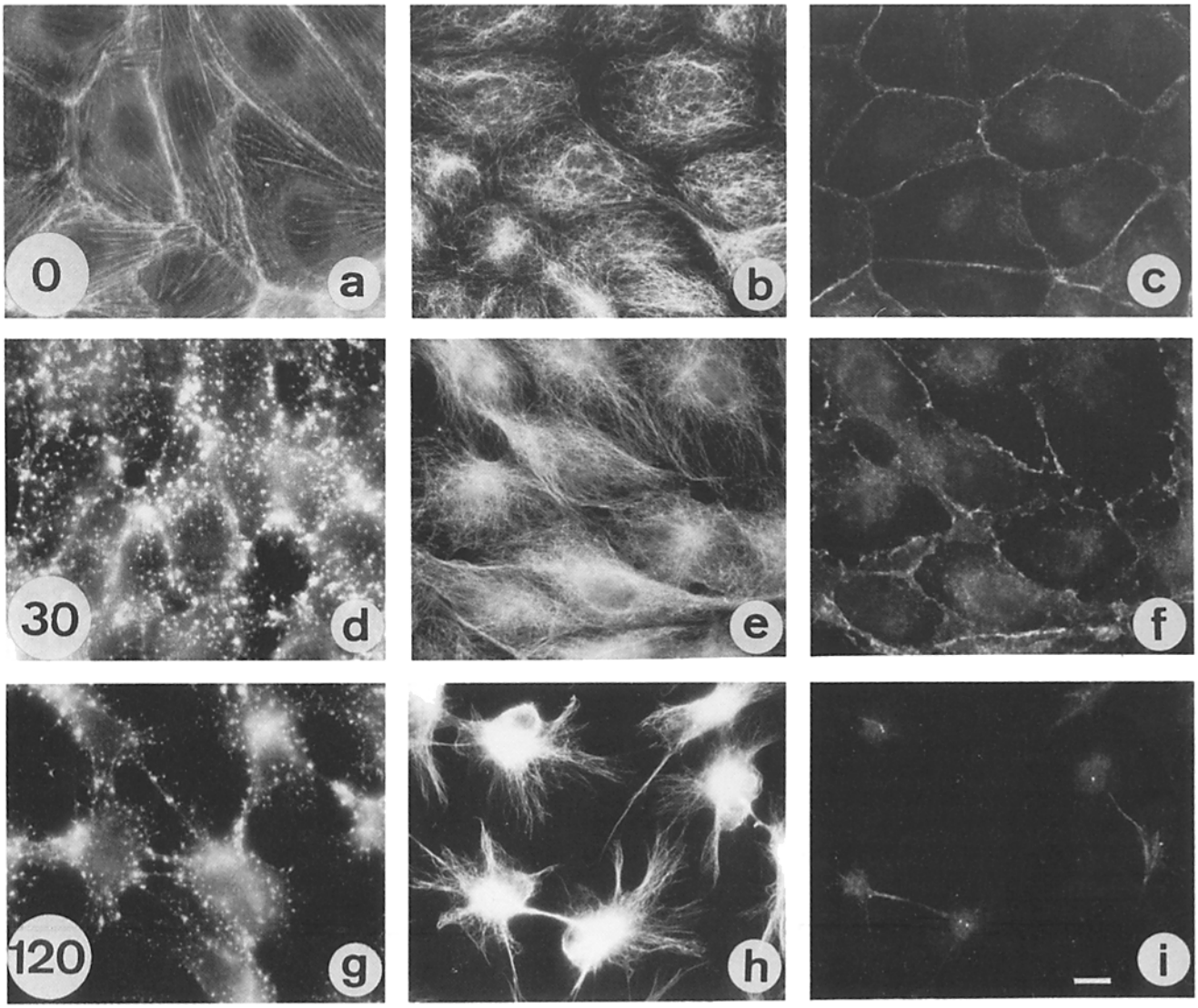

Figure 7. Disruption of microfilaments by cytochalasin D leads to microtubule reorganization. Subconfluent cells were fixed before or after various times of incubation in cytochalasin D. Numbers (*left*) indicate the time in minutes of incubation of cells in cytochalasin. The cells were stained for actin (*left*), tubulin (*center*), and for uvomorulin (*c* and *f*) or centrioles (*i*). Note the disruption of junctions after 30 min of exposure to cytochalasin. Bar, 10 μm.

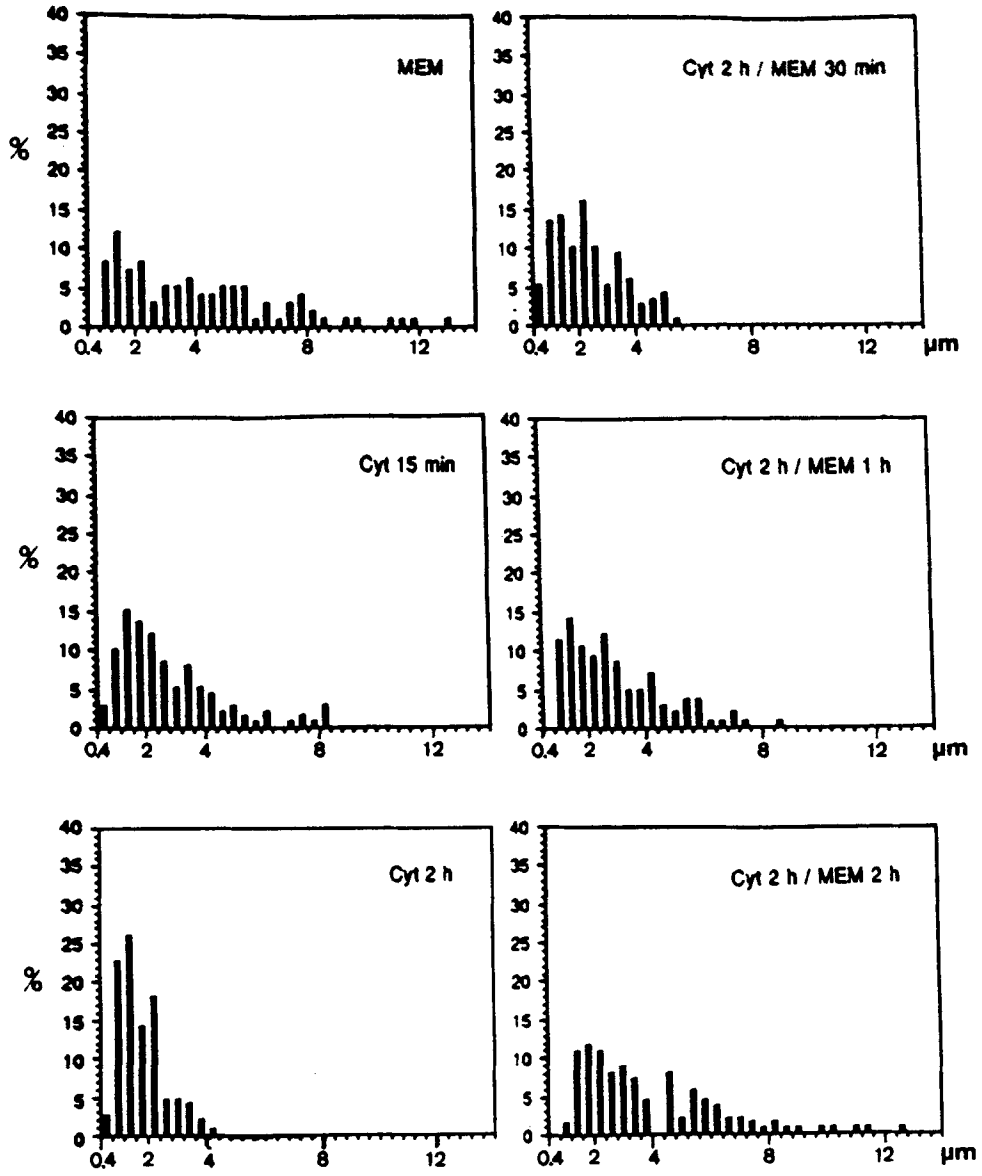

Figure 8. Microfilament disruption leads to reversal of centrosome splitting. The distance between centrioles was determined before (*MEM*), after incubation in cytochalasin D (*Cyt 15 min*; *Cyt 2 h*), and after reversion to normal medium for 30 min (*Cyt 2 h/MEM 30 min*), 1 h (*Cyt 2 h/MEM 1 h*) and 2 h (*Cyt 2 h/MEM 2 h*). Abscissa and ordinate are as described in the legend to Fig. 5.

lished, and the microtubule pattern was similar to that observed in untreated cells (Fig. 4 *j*). These results strongly suggest that centriole separation occurs in response to the establishment of cell junctions.

Role of Microfilaments in Centriole Localization and Microtubule Organization in Subconfluent Cells

Isolated MDCK cells showed a specific organization of their actin network. There was no extensive array of stress fibers, and most of the polymeric actin seemed to be organized in a more or less complete ring surrounding the nucleus, sometimes extending towards the cell periphery (Fig. 6 *b*). In subconfluent cells, stress fibers appeared in the basal domain and a ring structure delineating cell borders assembled (Fig. 7 *a*). This ring probably corresponded to microfilaments interacting with adherens junctions (for review, see Edelman, 1985; Volberg et al., 1986). Dots (microvilli) were also visible in the apical domain (Drenckhahn and Dermietzel, 1988). 5 min after cytochalasin addition, some stress fibers were still present and by 15 min only patches of actin remained at the cell periphery (not shown). After 30 min, the actin network was completely disorganized (Fig. 7, *d–g*) whereas cell surface staining with antiuvomorulin antibodies was still quite regular around many cells (Fig. 7 *f*). After 2 h, the cells had changed their shape dramatically, and uvomorulin was found only at contact points between cell projections (not shown, but see Fig. 7, *g*, *h*, and *i* for cell shape).

The centrioles started to move towards each other before any apparent disruption of the junctions. The population of cells having centrioles separated by <2 µm increased from 25 to 41% as soon as 15 min after cytochalasin addition (Fig. 8). After 30 min and 2 h in cytochalasin, centrioles closer than 2 µm were observed in 56% (not shown) and in 65% of the cells (Fig. 8), respectively. After removal of cytochalasin D from the medium, the centrioles moved away from each other over a period of 2 h (Fig. 8). During this reversal, a ring of actin reappeared at the same time as the junctions started to reassemble (not shown). The stress fibers on the basal side of the cells became visible 1 h after removal of the drug, and the cells lost their arborized appearance. Normal

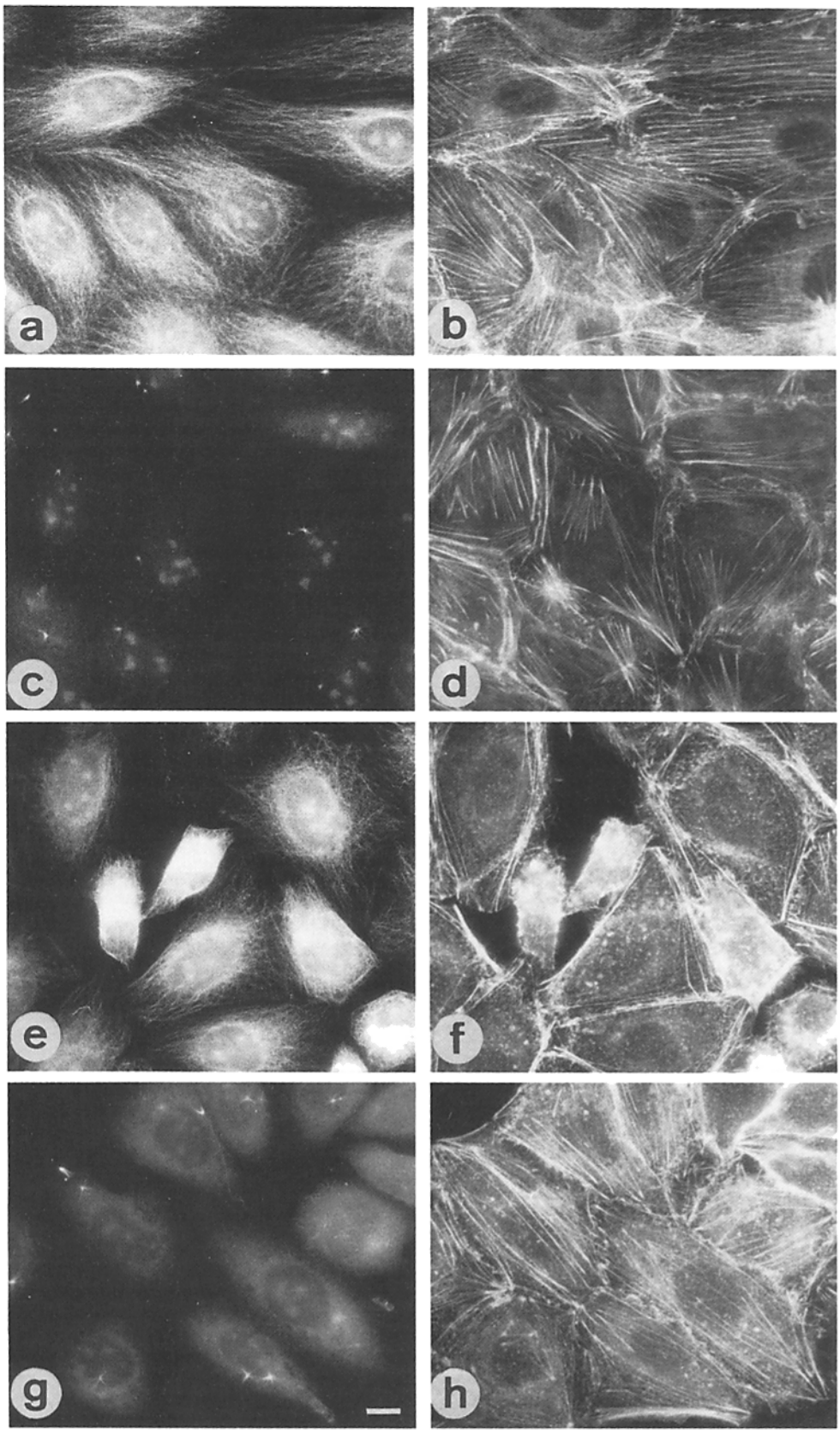

Figure 9. Effect of 33 μM nocodazole on microtubules and microfilaments in MDCK cells cultured in normal and low calcium medium. Cells were fixed with glutaraldehyde and double stained for microtubules (*left*) and actin (*right*). Some subconfluent cells were fixed before (*a* and *b*) or after 2 h of incubation in nocodazole (*c* and *d*). Other cells were treated for 2 h by low calcium medium either alone (*e* and *f*) or with the simultaneous addition of nocodazole (*g* and *h*). Bar, 10 μm.

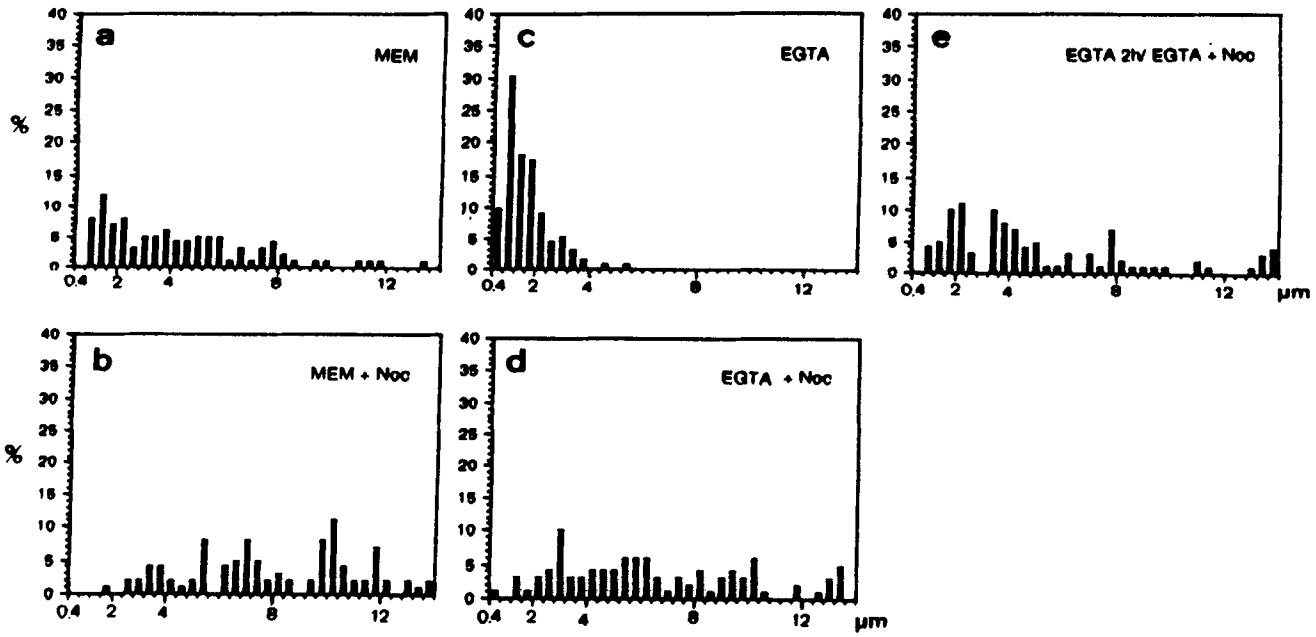

Figure 10. Reversal of centrosome splitting requires microtubules. The centrioles move back together after 2 h of incubation of the cells in low calcium medium (*a* and *c*). This does not happen in cells incubated in nocodazole (*b* and *d*). In cells incubated for 2 h in low calcium medium, and exposed for 1 h to nocodazole in the same medium, the centrioles split again (*e*). Abscissa and ordinate are as described in the legend to Fig. 5.

cell morphology was reestablished 2 h after cytochalasin D removal and the frequency of cells with centrioles closer than 2 μm returned to 25%.

Actin disorganization had an interesting effect on the microtubule pattern. As early as 30 min after cytochalasin addition the microtubule network became fuzzy and the appearance of asters was evident in many cells (Fig. 7 *e*). 2 h after addition of the drug most subconfluent cells had an astral shape (Fig. 7, *g–h*), as already reported (Miranda et al., 1974; Schliwa, 1982; Meza et al., 1982) and the microtubules were all radiating from the region of the cell center which contained the two centrioles (Fig. 7, *h–i*).

Therefore, it appears that there is a strict temporal correlation between microfilament disassembly, movement of the centrioles towards the nucleus and reorganization of the microtubule network in a radial array. These phenomena are all readily reversed after reassembly of the actin network indicating that, in cells becoming polarized, the actin microfilament network plays an important role both in centriole separation and reorganization of the microtubule network.

Centrioles are Moved Towards Each Other by Microtubules

Extensive microtubule depolymerization by nocodazole (Fig. 9, *c* and *g*), increased the average intercentriolar distance in subconfluent cells; the frequency of cells showing centrioles separated by <2 μm dropped from 27% in normal medium (Fig. 10 *a*) to only 1% following incubation in nocodazole (Fig. 10 *b*). As already mentioned, incubation of subconfluent cells in low calcium medium resulted in reversal of centriole separation (Fig. 10 *c*). This did not occur at all when the cells were transferred to low calcium medium containing nocodazole. Under this condition, instead, the centrioles moved further apart (Fig. 10 *d*).

These results clearly showed that in subconfluent cells exposed to low calcium medium, centrioles did not move back together in the absence of intact microtubules. We then asked whether the centrioles that were located close to each other in cells cultured in low calcium medium for 2 h, would separate again after microtubule disruption. As shown in Fig. 10 *e*, the centrioles did in fact separate, the intercentriolar distance becoming homogeneously distributed between the extreme values of 0.8 and 13 μm. The same result was obtained in isolated cells cultured in normal medium and exposed to nocodazole.

Centrioles Can Separate in the Absence of Microtubules and Microfilaments

The potential role of actin in the mechanism of centriole separation in the absence of microtubules was investigated in subconfluent cells exposed to low calcium medium for 2 h. This produced a population in which centrioles were close to each other (75% of the cells had centrioles closer than 2 μm, Fig. 11, *a* and *b*). This was more convenient than isolated cells for quantitating the data since there were many more cells in each field. Subsequently, both cytochalasin D and nocodazole were added to the low calcium medium and the intercentriolar distance measured. After 1 h, extensive separation of centrioles had occurred (Fig. 11 *c*). This shows that centrioles can separate in the absence of both microtubules and microfilaments. We assume that this is because of random motion of the freed centrioles.

Discussion

The exact role of centrioles in microtubule nucleation is still unclear (Wheatley, 1982; Keryer et al., 1984; Euteneuer and Schliwa, 1986). In many cells, microtubules are nucleated

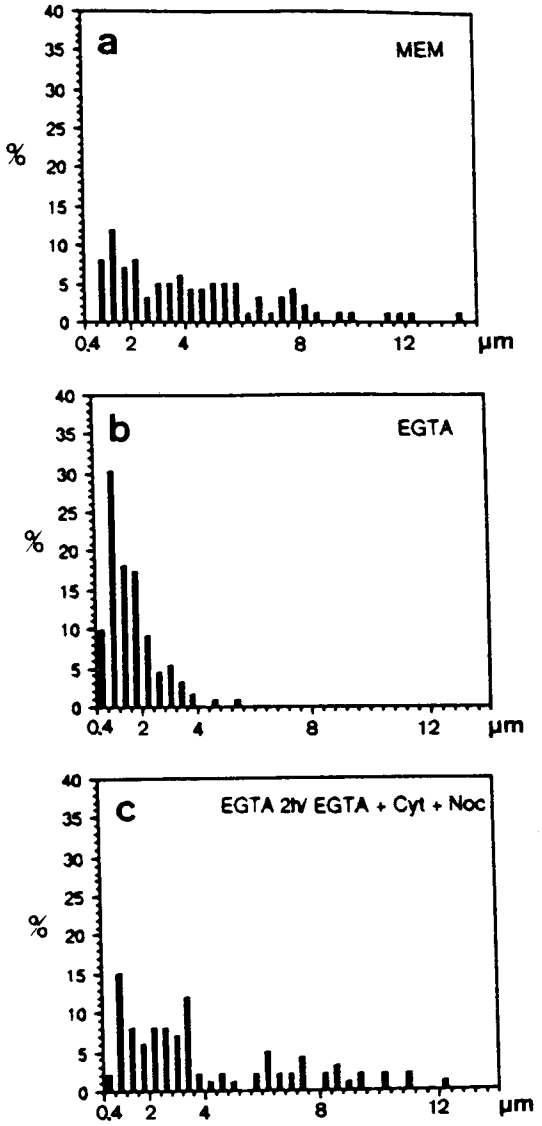

Figure 11. In the absence of microtubules and microfilaments, centrioles show a random position independent of cell contacts. The distance between centrioles was determined for 120 cells incubated in normal medium (*a*), exposed to low calcium medium for 2 h (*b*), and incubated in low calcium medium containing cytochalasin D and nocodazole for 1 h after a 2-h preincubation in low calcium medium (*c*).

by some amorphous material associated with the centrioles, the pericentriolar material. However, there are cases where the nucleating material is not found associated with centriole cylinders (Mazia, 1984; Tassin et al., 1985; Karsenti and Maro, 1986). It is still unclear how important the centriole itself is in microtubule nucleation in MDCK cells. Although it does not nucleate many microtubules at steady state in subconfluent cells, it does so after microtubule depolymerization by nocodazole and drug removal (Bré et al., 1987). It is likely that the microtubule-nucleating material is in part associated to the centriole cylinders and in part dispersed in the cytoplasm, or associated with other cytoskeletal elements. In any case, for this study, we considered the centrioles as convenient markers to follow the movement of the nucleating material.

Centrioles Movements during Polarization

The behavior of centrioles in MDCK cells is quite different from what it is in fibroblasts. In isolated MDCK cells, the centrioles are close to each other and to the nucleus although usually not as tightly associated as in fibroblasts or in lymphoid cells. The two centriole cylinders isolated from human lymphoid cells are linked by a specific structure (Bornens et al., 1987). Such a structure was not evident in MDCK cells. In clusters of 5–10 MDCK cells, the centrioles move apart only in those cells that are in the center of the clusters (not shown). In peripheral cells, centrioles are always close to each other. This observation suggests that the establishment of multiple cellular junctions is closely coupled to the separation of centrioles. In subconfluent cells forming a homogeneous monolayer, the intercentriolar distance varies between 0.4 and 13 μm. This suggests that under these conditions, the two centrioles are able to move both away from and towards each other. This movement of the centrioles may be because of the fact that subconfluent cells are engaged in the process of polarization and the differences we see merely reflect the various stages in this process. As shown by immunofluorescence and the EM study, in fully polarized MDCK cells the centrioles are located close to each other and near the apical plasma membrane as in many other epithelial cells (Dustin, 1984). Therefore, we think that centrioles separation in subconfluent MDCK cells represents an intermediary event in

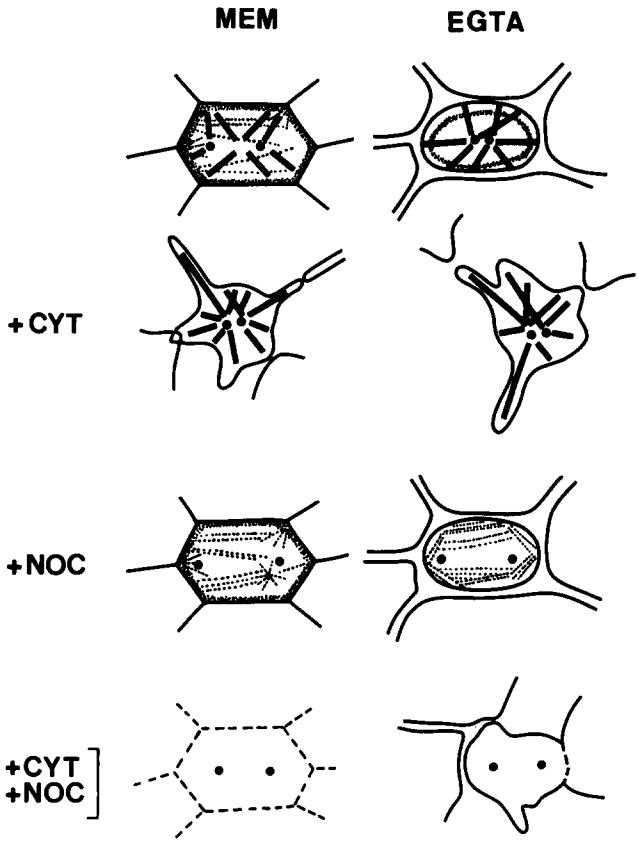

Figure 12. Schematic diagram of the position of centrioles in relation to cell shape, and cell contacts. Centrioles, solid black circles; microtubules, thick lines; microfilaments, broken lines; effect of cytochalasin (*CYT*) and/or nocodazole (*NOC*).

the migration of centrioles towards the apical membrane during the polarization process.

Intercellular Junctions and Centrioles Separation

Calcium removal is a widely used method to disrupt the junctions of epithelial cells (Hoi-Sang et al., 1979; Martinez-Palomo et al., 1980; Gumbiner and Simons, 1986; Mattey and Garrod, 1986; Vega-Salas et al., 1987 and 1988). This treatment results in the movement of centrioles towards each other. We propose that the assembly of intercellular junctions is one of the initial events that is required for centrioles separation. This is further supported by the observations that centrioles clearly separate during or slightly after junction reassembly when the cells are transferred back to normal medium, under conditions where the external calcium is at a physiological concentration. Low calcium medium has also been reported to induce a reorganization of microfilaments and intermediate filaments. It appears however that this is a consequence of junction disruption rather than a direct effect of divalent cation chelation on these cytoskeletal structures (Volberg et al., 1986; Green et al., 1987). This is supported by our observation that the actin organization in subconfluent cells cultured in low calcium medium is very similar to that observed in isolated cells cultured in normal medium. Hence, by using low calcium medium we produce cells that closely resemble isolated cells (lacking junctions). Since this event happens in <2 h, this system is more convenient than an isolated cell system. Moreover, we can follow what happens during junction reassembly in normal medium. A definitive proof of the initial and specific role of epithelial cell junction assembly in centrioles separation will require the inhibition of assembly of specific junctions with antibodies. Unfortunately, the only antibody we could have used to do such experiments (Gumbiner and Simons, 1986; Gumbiner et al., 1988) did not have sufficient inhibitory properties on junction assembly to provide convincing results (this antibody only slowed down the kinetics of junction assembly).

Control of Centrioles Movements by Microfilaments and Microtubules during Polarization

In isolated cells, or in subconfluent cells incubated in low calcium medium, the centrioles are located close to each other and to the nucleus. We propose that in the absence of cell-cell interactions, microtubules (probably the few that are nucleated by the centrioles) push the centrioles towards the cell center according to a mechanism previously proposed by Euteneuer and Schliwa (1985): the equilibrium position of centrioles would be reached when "all the microtubules that they nucleate are minimally bent and are of more or less equal length" (see Fig. 12, *top right*). Such a mechanism could also function for other nucleating sites not associated with centrioles (which we cannot follow here). Upon the establishment of extensive cell-cell interactions, a change in the actin network occurs, probably because of the assembly of intercellular junctions (and their subsequent interaction with microfilaments). Under this new condition, we argue that the actin network would then pull the nucleating material, either directly or indirectly through the microtubules, towards the cell periphery leading to centriole separation (Fig. 12, *top left*). In the absence of microfilaments, centrioles move back together, and, in the absence of microtubules, they split apart in all cells (Fig. 12). These observations give an impression of how the nucleating material is relocalized during the establishment of a confluent monolayer of epithelial cells. However, how the nucleating material is finally moved up towards the apical plasma membrane remains to be investigated. This movement seems also to require actin in epithelia (Lemullois et al., 1988).

In parallel to this work, we have accomplished an extensive study of microtubule reorganization during the polarization of MDCK cells grown on filter supports (Bacallao et al., 1989). In polarized cells, 90% of the microtubules are oriented with their minus end in the apical region of the cells and their plus end in the basal domain. This observation fits well with the position of the centrioles in the apical domain (close to the minus end of microtubules). This final orientation is preceded by a cellular stage in which microtubules are arranged more or less parallel to the long axis of the cell and to the substratum. Here, we have also observed this special organization and shown that it is lost when microfilaments are disrupted (Fig. 7, *e* and *h*). The microtubules thus assume a radial organization at the same time as the centrioles move back together in the cell center. This strongly suggests that the organization as well as the orientation of microtubules are determined through the localization of nucleating material. The position of this material is itself determined in part by the state of the actin network that would be modulated by the status of cellular junctions assembly. The challenge is now to find out how all these elements interact at the molecular level to produce the morphogenetic events leading to microtubule orientation and final polarization of the cytoplasm of epithelial cells.

In conclusion, we favor the idea that the establishment of cell junctions leads to a rearrangement of the actin network that, in turn, pulls the centrioles and other microtubule nucleating material towards the cell periphery. This movement is counteracted by an elongation of the nucleated microtubules that tend to push the nucleating material towards the cell center. Together, these two opposing forces could allow a precise positioning of the centrioles during the polarization process.

We thank K. Simons, R. Bacallao, and C. Dotti for continuous discussions and critical reading of the manuscript. We thank M. Bornens for the gift of the monoclonal anticentrosome antibody, Jan De Mey for the gift of the rabbit antitubulin, R. Pepperkok for help with the computer program, R. Back for excellent technical assistance with electron microscopy, and A. Walter for help in preparing this manuscript.

Received for publication 27 June 1989 and in revised form 6 December 1989.

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