Accumulation of a Microtubule-binding Protein, pp170, at Desmosomal Plaques

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Abstract. The establishment of epithelial cell polarity correlates with the formation of specialized cell–cell junctions and striking changes in the organization of microtubules. A significant fraction of the microtubules in MDCK cells become stabilized, noncentrosomally organized, and arranged in longitudinal bundles in the apical-basal axis. This correlation suggests a functional link between cell–cell junction formation and control of microtubule organization. We have followed the distribution of pp170, a recently described microtubule-binding protein, during establishment of epithelial cell polarity. This protein shows the typical patchy distribution along microtubules in subconfluent fibroblasts and epithelial cells, often associated with the peripheral ends of a subpopulation of microtubules. In contrast to its localization in confluent fibroblasts (A72) and HeLa cells, however, pp170 accumulates in patches delineating the regions of cell–cell contacts in confluent polarizing epithelial cells (MDCK and Caco-2). Double immunolocalization with antibodies specific for cell–cell junction proteins, confocal microscopy, and immunoelectron microscopy on polarized MDCK cells suggest that pp170 accumulates at desmosomal plaques. Furthermore, microtubules and desmosomes are found in close contact. Maintenance of the desmosomal association of pp170 is dependent on intact microtubules in 3-d-old, but not in 1-d-old MDCK cell cultures. This suggests a regulated interaction between microtubules and desmosomes and a role for pp170 in the control of changes in the properties of microtubules induced by epithelial cell–cell junction formation.

IMPORTANT events in the establishment of cellular polarity include the reorganization of the cytoskeleton. The spatial arrangement of cytoplasmic organelles and intracellular membrane traffic, for example, directly correlate with the organization of the interphase microtubule network and have been studied in a number of cells. Well established cell systems for studying epithelial cell morphogenesis are the MDCK and Caco-2 cell lines which can differentiate into polarized transporting epithelia in culture (for reviews see Rodriguez-Boulan and Nelson, 1989; Simons and Wandinger-Ness, 1990).

Upon formation of a polarized MDCK cell monolayer, microtubules, which nucleate preferentially from a perinuclear region in single cells, reorganize into bundles running parallel to the apical-basal axis and no longer originate from this perinuclear microtubule organizing center (Bré et al., 1990; Bacallao et al., 1989). Concomitant with microtubule reorganization, the centrosomes split and migrate to the apex of the polarized cells (Buendia et al., 1990). In addition, with the formation of cell–cell contacts microtubules become stabilized. The average half-life of microtubules, which in single MDCK cells is comparable to that measured in fibroblasts, increases upon cell–cell contact formation in MDCK cells (Pepperkok et al., 1990; Bré et al., 1990), while it remains unchanged in confluent fibroblasts (Pepperkok et al., 1990; Wadsworth and McGrail, 1990). The temporal correlation between the increase in microtubule stability and the establishment of cell–cell junctions suggests a role for junction formation in signaling induction of microtubule reorganization.

The factors that induce and regulate the stabilization and reorganization of microtubules upon cell–cell contact formation and establishment of cell polarity are so far unknown. It may be speculated, however, that likely candidates for factors playing a role in the control of microtubule organization in polarizing cells are proteins with a peripheral localization and the capacity to interact with microtubules in a regulated manner. A microtubule-binding protein of M_r 170,000 (pp170) which accumulates at microtubule plus ends and whose binding to microtubules in vitro is regulated by phosphorylation has recently been identified in HeLa cells (Rickard and Kreis, 1990, 1991). In this report we characterize the distribution of pp170 in polarizing epithelial cells and show that pp170 associates with desmosomal plaques upon cell–cell contact formation. These results suggest that desmosomes may capture microtubules and play an important role in regulating microtubule dynamics. Furthermore, pp170 could be involved in the temporal correlation between establishment of cell-cell junctions and microtubules reorganization during epithelial cell polarization.

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Materials and Methods

Cell Culture

Human intestinal epithelial cells, Caco-2 (kindly provided by Dr. H.-P. Hauri, Basel, Switzerland), MDCK, and HeLa cells were grown in MEM, MDBK (Madin-Darby bovine kidney) cells in DME, and A72, tumor-derived dog fibroblast cells (Binn et al., 1980), in L-15 (Leibovitz) medium. All media were supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), as well as 5% (MDCK), 10% (MDBK, A72, HeLa), or 20% (Caco-2) FCS at 37°C in a humidified atmosphere containing 5% CO_2. The HeLa and Caco-2 cell media contained 1% nonessential amino acids; and the HeLa cell medium was supplemented with 1 mM Na-pyruvate. Cells for conventional immunofluorescence were plated on glass coverslips. Polarized MDCK cells were obtained by plating cells at a density of 3.4×10^5 cells/cm^2 on 10-mm diameter transparent filter inserts (Anopore inorganic membrane filters; Nunc, Wiesbaden, Germany) placed in Nunc six-well plates and incubating them for 4–5 d under the conditions indicated above but with medium containing 10% FCS. 2.5 ml medium was added at the basal side and 0.5 ml at the apical side.

Drug Treatments

MDCK cells grown on coverslips for 1 ("young cells") or 3 d ("old cells") to produce small islands or confluent monolayers, respectively, were treated with 33 μM nocodazole (Sigma, Deisenhofen, Germany) for 4 h at 37°C to depolymerize microtubules. Vinblastine (Sigma) or taxol (gift of Dr. M. Suffness, Natural Product Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD) were applied at 10 μM also for 4 h at 37°C.

Antibodies

Murine mAbs against α- and β-tubulin were obtained from Amersham (Braunschweig, Germany), against desmosomal protein, clone ZK-31 (Lang et al., 1986) from Sigma, against desmoplakin 1 and 2 (clone DP1+2 −2.15) from Boehringer (Mannheim, Germany), and against vinculin from Boehringer. Rabbit antibodies against pp170 (α-pp170) were affinity-purified as described (Rickard and Kreis, 1990, 1991). Rabbit antibodies against α-tubulin were characterized elsewhere (Kreis, 1987). In some experiments, 4D3, a murine mAb raised against pp170 was used (Rickard and Kreis, 1991). Secondary antibodies were rhodamine- or fluorescein-labeled goat-anti-rabbit or anti-mouse IgG (Kreis, 1986).

Immunofluorescence Labeling

Cells were either directly fixed or preextracted with 0.5% Triton X-100 in microtubule-stabilizing buffer (PHEM: 60 mM Pipes, 25 mM Hepes, 1 mM EGTA, 1 mM Mg-acetate, pH 6.9; Schliwa et al., 1981) four times for 3 s or once for 2 min, and then fixed for 8 min in methanol, followed by 4 min in acetone, both at −20°C. Alternatively, they were fixed for 10 min at room temperature in 1.75% paraformaldehyde in PHEM, washed three times for 5 min with PHEM and permeabilized for 90 s in ice-cold methanol. After 5 min washes, two each in TBS (10 mM Tris, 154 mM NaCl, pH 7.6) and TBS/BSA (TBS containing 0.05% BSA and 0.02% NaN_3), cells on coverslips were incubated with the primary antibodies overnight at room temperature, washed three times for 10 min with TBS/BSA, incubated with secondary antibodies for 1 h at 37°C and washed three times for 10 min with TBS/BSA. Cells were then postfixed for 30 min with 3% formaldehyde in PBS, quenched for 15 min with 50 mM NH_4Cl in PBS, and mounted with spacers (plastic strips, ~100 μm thick) in 50% glycerol in PBS (Bacallao et al., 1989) containing as an antifading reagent 100 mg/ml 1,4-diazabicyclo-(2,2,2)octane (Sigma) as described (Langanger et al., 1983).

Fluorescence Microscopy

Conventional epifluorescence microscopy was performed using a Zeiss Axiophot and photographs were taken on Kodak Tmax P3200 film. Image acquisition and processing on the modular confocal microscope constructed at EMBL was as described (Bré et al., 1990).

Immunogold Labeling for EM

MDCK cells grown on coverslips were processed as described for indirect immunofluorescence labeling and incubated overnight with rabbit α-pp170. Three washes of 10 min with TBS/BSA containing 0.2% coldwater fish skin gelatin (FSG; Sigma) were followed by incubation with goat antirabbit antibodies coupled with 1-nm gold (Auroprobe One Gar; Amersham) or 10-nm colloidal gold (AuroProbe EM GAR G10; Amersham) for 2 h at 37°C. Further washes included 3 × 10 min in the same buffer and 3 × 5 min in PBS. After postfixation in 2% glutaraldehyde, 0.2% tannic acid in PBS for 30 min, washing in PBS and H_2O (3 × 5 min each), 1-nm gold conjugates were enhanced with silverlactate for 12 min under red light, according to the method of Danscher (1981), modified according to Namork and Heier (1989). Extensive washing with H_2O was followed by osmification for 10 min on ice with 0.5% OsO_4 in H_2O. Further processing of the specimen was by standard methods.

Gel Electrophoresis and Immunoblotting

Cells grown on 500-cm^2 plastic dishes (Nunc) were washed with PBS and PHEM, scraped into a minimal volume of ice-cold PHEM using a rubber policeman, and pelleted for 10 min in a Biofuge A (Heraeus, Osterode, Germany) at 13,000 rpm. Cells were resuspended in a volume equal to the pellet of PHEM containing 1 mM PMSF, 2 mM DTT, and 40 μg/ml of cytochalasin D, aprotinin, leupeptin, and pepstatin A (all from Sigma). Cells were then lysed in twice this volume of 95°C Laemmli sample buffer, centrifuged for 30 min at 4°C, 200,000 *g*, and supernatants used for SDS-PAGE. A cDNA clone partially encoding HeLa pp170 was expressed in bacteria in the vector pUEX1 as a fusion protein with β-galactosidase and isolated as inclusion bodies. Urea-solubilized inclusion bodies were used for probing with antibodies by immunoblotting. Proteins were separated on 0.75-mm-thick 8% polyacrylamide gels run at constant 180 V and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) using a Trans-Blot SD semidry blotter (Bio-Rad Laboratories, Munich, Germany) at 25 V for 1 h. Immunolabeling was done as described before (Rickard and Kreis, 1990).

Results

Localization of pp170 in Epithelial Cells

The affinity-purified rabbit antibodies (α-pp170) and the murine mAb 4D3 raised against HeLa pp170 were tested by immunoblotting on lysates of human HeLa and Caco-2, as well as on dog A72 and MDCK cells, which are nonpolarizing and polarizing cell lines of the same species. α-pp170 recognizes pp170 in all four cell lines (Fig. 1 *b*, lanes *2–5*). 4D3 reacts with pp170 in HeLa and Caco-2 cell lysates (Fig. 1 *c*, lanes *2* and *4*), but does not crossreact with pp170 in A72 and MDCK cells (Fig. 1 *c*, lanes *3* and *5*). The signal for pp170 detected by immunoblotting is consistently weaker in Caco-2 and MDCK (Fig. 1 *b*, lanes *4* and *5*), than in HeLa and A72 cells (Fig. 1 *b*, lanes *2* and *3*), indicating that pp170 is less abundant in the polarizing cells of both species. 4D3 does not react with the bacterial fusion protein of the partial cDNA clone of pp170 which was used to affinity purify α-pp170 (Fig. 1, *b* and *c*, lane *1*), indicating that 4D3 and the rabbit antibodies bind to different epitopes on pp170.

Since the changes in microtubule organization that occur during polarization are much better characterized in MDCK than in Caco-2 cells, we chose to follow the distribution of pp170 during this process using MDCK cells. The distribution of pp170 was analyzed both in dog fibroblastic A72 and epithelial MDCK cells at various stages of confluency (polarization) and compared (Fig. 2). Double immunofluorescence labeling with α-pp170 and the monoclonal antitubulin antibodies reveals a patchy distribution of pp170 along microtubules in confluent (Fig. 2 *a,a'*) or subconfluent (Fig. 2 *b,b'*) cultures of A72 and MDCK (Fig. 2 *c,c'*) cells, comparable to the pattern found in HeLa cells (Rickard and Kreis, 1990). In addition, significant labeling of pp170 can be detected in the area of the centrosomes (e.g., Fig. 2 *a*,

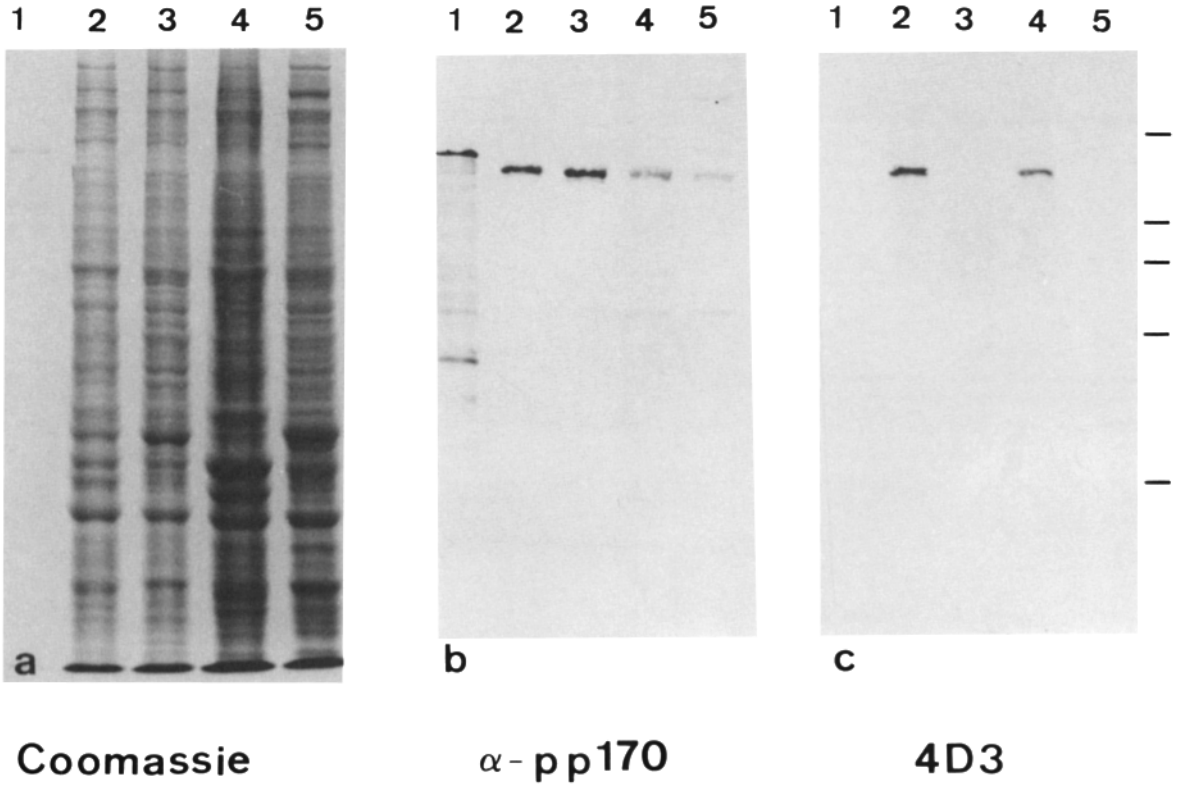


Figure 1. Specificity of the antibodies against pp170 on fibroblasts and epithelial cells. pp170 fusion protein (lanes *1*) and total lysates of HeLa (lanes *2*), A72 (lanes *3*), Caco-2 (lanes *4*), and MDCK (lanes *5*) cells were prepared, separated by SDS-PAGE, and stained with Coomassie brilliant blue (*a*) or immunolabeled with rabbit α-pp170 (*b*) or mouse 4D3 (*c*) as described in Materials and Methods. Molecular masses of marker proteins are indicated (200, 116, 97, 66, 43 kD).

arrows). This centrosomal labeling has been found in all the cell lines investigated. pp170 remains associated with the centrosomes of polarized MCDK cells, even though the capacity of these centrosomes to nucleate cytoplasmic microtubules is diminished (Bré et al., 1987). In contrast, in MDCK cells which have established cell–cell contacts, pp170 accumulates in patches delineating the region of cellular junctions (Fig. 2 *c*). Alignment of such patches of pp170 are, however, not detected in confluent A72 cells (Fig. 2 *a*) and HeLa cells (not shown) in areas of cell–cell contacts. A similar pattern has also been detected in the human epithelial cells, Caco-2, both by labeling with α-pp170 (see Fig. 4 *b*) and 4D3 (not shown). The localization of pp170 to desmosomes is independent of the method of cell fixation. Preextraction of cells with Triton X-100 for up to 3 min before fixation, however, resulted in clearer pictures (Fig. 2), than when cells were directly fixed (for details see Materials and Methods) either with methanol/acetone (Fig. 3 *a*) or with paraformaldehyde/methanol (Fig. 3 *b*).

pp170 Colocalizes with Desmosomes in Polarized Epithelial Cells

The accumulation of pp170 in cell–cell contacts was further investigated by double labeling with antibodies specific for junctional proteins (Fig. 3 and 4). pp170 colocalizes with desmosomes as detected by a mAb against desmosomal protein (or desmoplakin, not shown) in MDCK (Fig. 3 and 4, *a*,*a'*), Caco-2 (Fig. 4, *b*,*b'*), and MDBK (Fig. 4, *c*,*c*) cells. Colocalization of pp170 with desmosomes was clearest in MDBK cells which display best separation of desmosomes.

The distribution of pp170 and desmosomal protein in polarized MDCK cells grown on filters was also investigated using confocal microscopy (Fig. 5). A clear colocalization of pp170 and the desmosomal marker can be found in the stereo reconstructions (Fig. 5, *a* and *b*). pp170 is predominantly located along the basolateral membranes (Fig. 5 *a*); the centrosomes, which are also labeled with α-pp170, are in the apex of the cell (Fig. 5 *a*, *arrows*). Superposition of both stereo images demonstrated virtually complete coincidence of pp170 with the desmosomal marker at the basolateral membrane (not shown).

Since plakoglobin is a component of both desmosomes, the zonula adherens and other intermediate junctions (for reviews see Kapprell et al., 1990; Schwarz et al., 1990), colocalization experiments were also performed using a mAb against vinculin, a marker protein of adherens junctions (for a review see Geiger et al., 1985). MDBK cells were used here since they have a well-defined zonula adherens and the vinculin-containing belt-like structure is clearly revealed (Fig. 4 *d'*). The flared speckles, which are not in focus, are focal contacts at the base of the monolayer. The structures labeled by α-pp170 (Fig. 4 *d*), are in the same plane of focus as the zonula adherens, but appear more spotty and do not colocalize with vinculin, indicating that pp170 is not associated with the zonula adherens. Furthermore, HeLa cells expressing uvomorulin (generous gift of Dr. R. Kemler, MPI, Freiburg, Germany) and accumulating

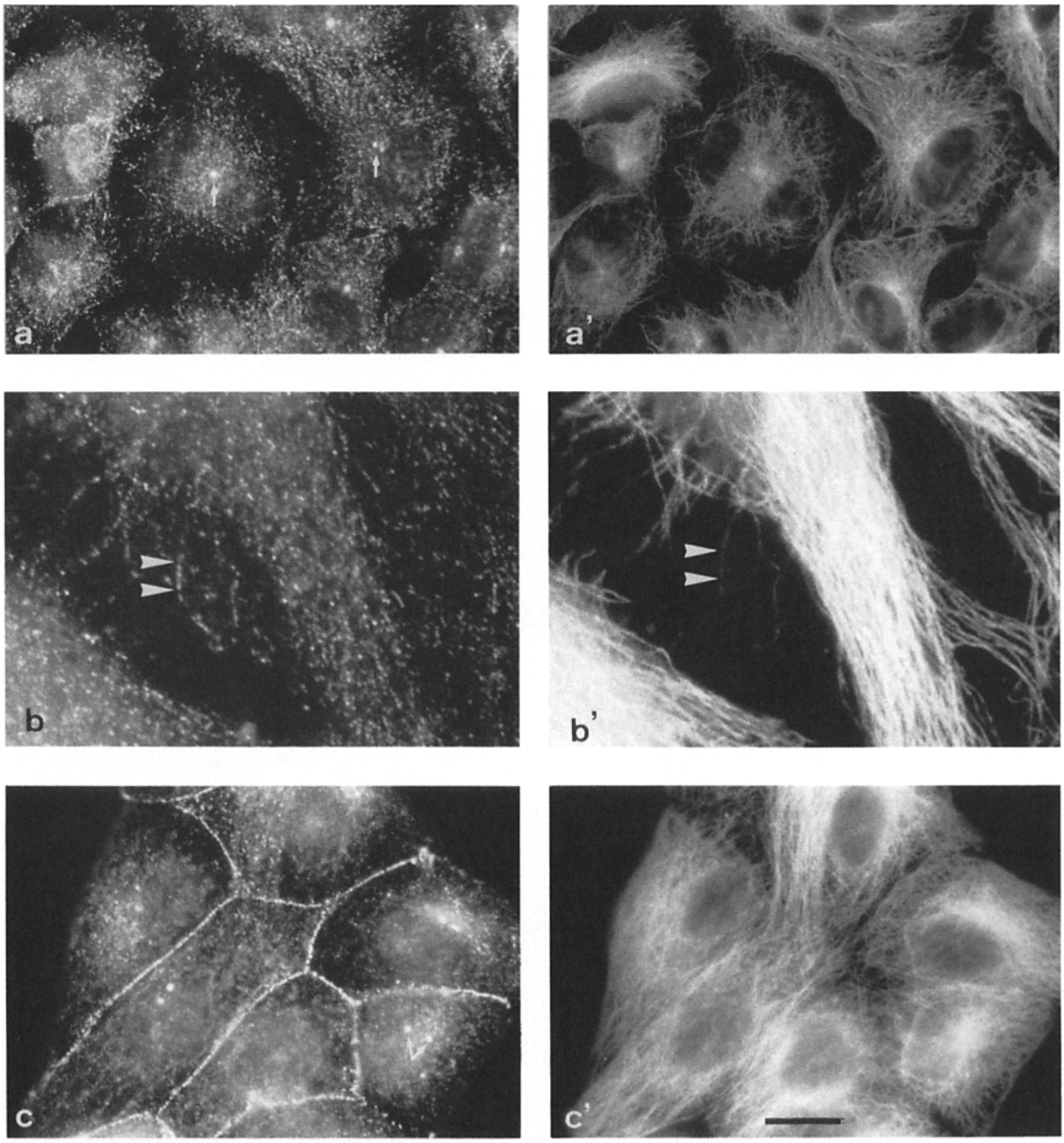


Figure 2. Immunofluorescence localization of ppl70 in fibroblasts and epithelial cells. Confluent (*a*,*a'*) or subconfluent (*b*,*b'*) A72 cells, and MDCK cells (*c*,*c'*) were fixed with methanol/acetone after preextraction with detergent as described in Materials and Methods. Cells were double labeled with rabbit α-ppl70 (*a–c*) and mouse anti-tubulin antibodies (*a'–c'*). Arrows indicate centrosomes; arrowheads show ppl70 localizing along a microtubule. Bar, 20 μm.

this protein in the region of cell–cell contacts do not show ppl70 accumulation in this area (not shown).

ppl70 Is Found on Microtubules and at Desmosomal Plaques by Immunoelectron Microscopy

To investigate the subcellular distribution of ppl70 on the EM level, a preembedding labeling procedure was used on methanol/acetone-fixed MDCK cells (Geuens et al. 1983), since α-ppl70 labels aldehyde-fixed cells only weakly. Sites where primary antibodies had bound were visualized by labeling with secondary antibodies either coupled to 10-nm colloidal gold, or 1-nm colloidal gold which was then enhanced with silver. In ultrathin sections, 10-nm gold particles (Fig. 6 *a*) or silver grains (Fig. 6, *b* and *c*) are scattered along microtubules (Fig. 6 *a*) which is in clear contrast to

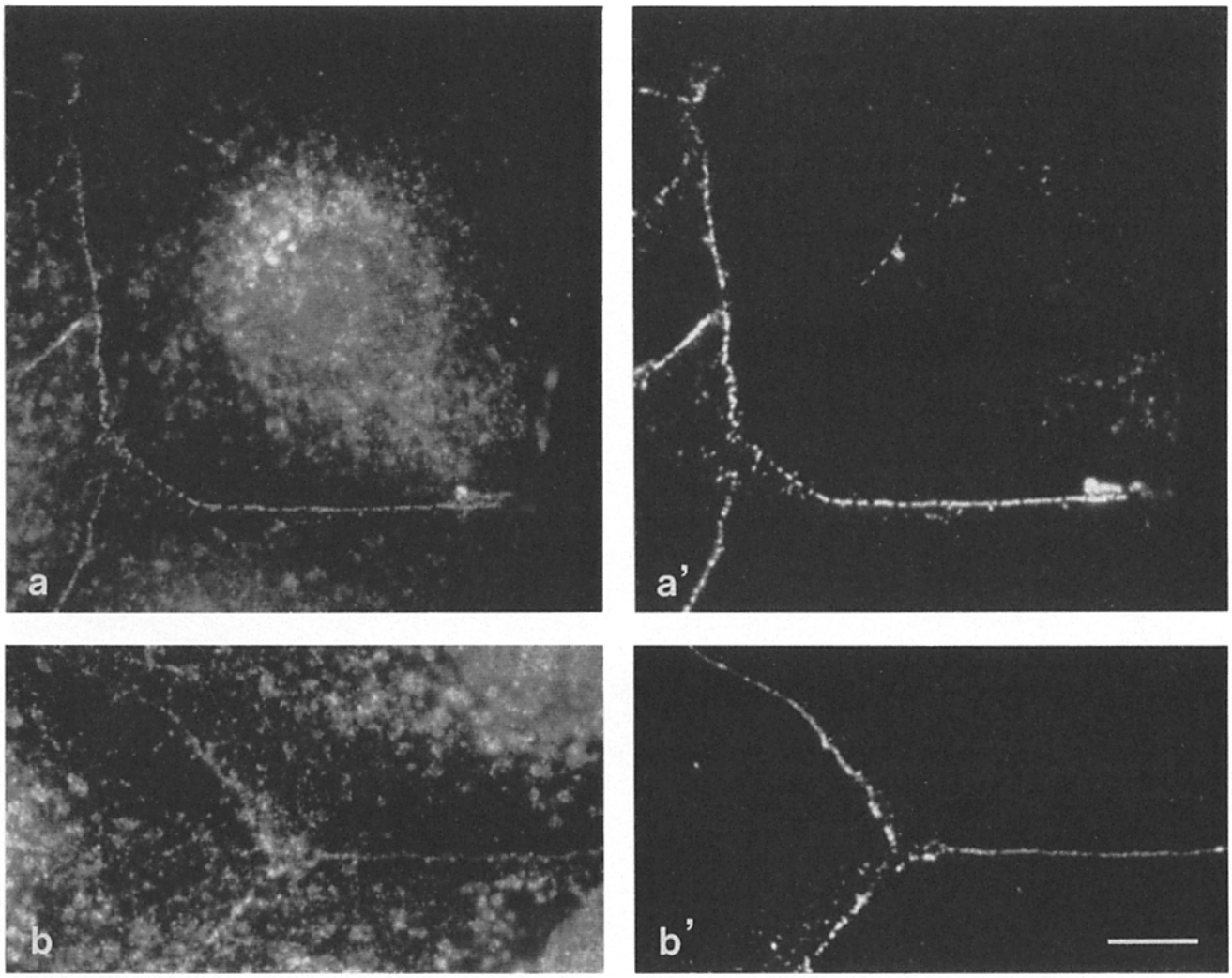


Figure 3. Localization of pp170 to desmosomes is independent of the fixation of MDCK cells. Subconfluent MDCK cells were fixed directly in methanol (*a*,*a'*) or paraformaldehyde methanol (*b*,*b'*) as described in Materials and Methods. Fixed cells were double labeled with rabbit α-pp170 (*a*,*b*) and mouse antidesmosomal protein (*a'*,*b'*). Association of pp170 with desmosomes is clearly visible in MDCK cells fixed according to either of the two protocols. Bar, 20 μm.

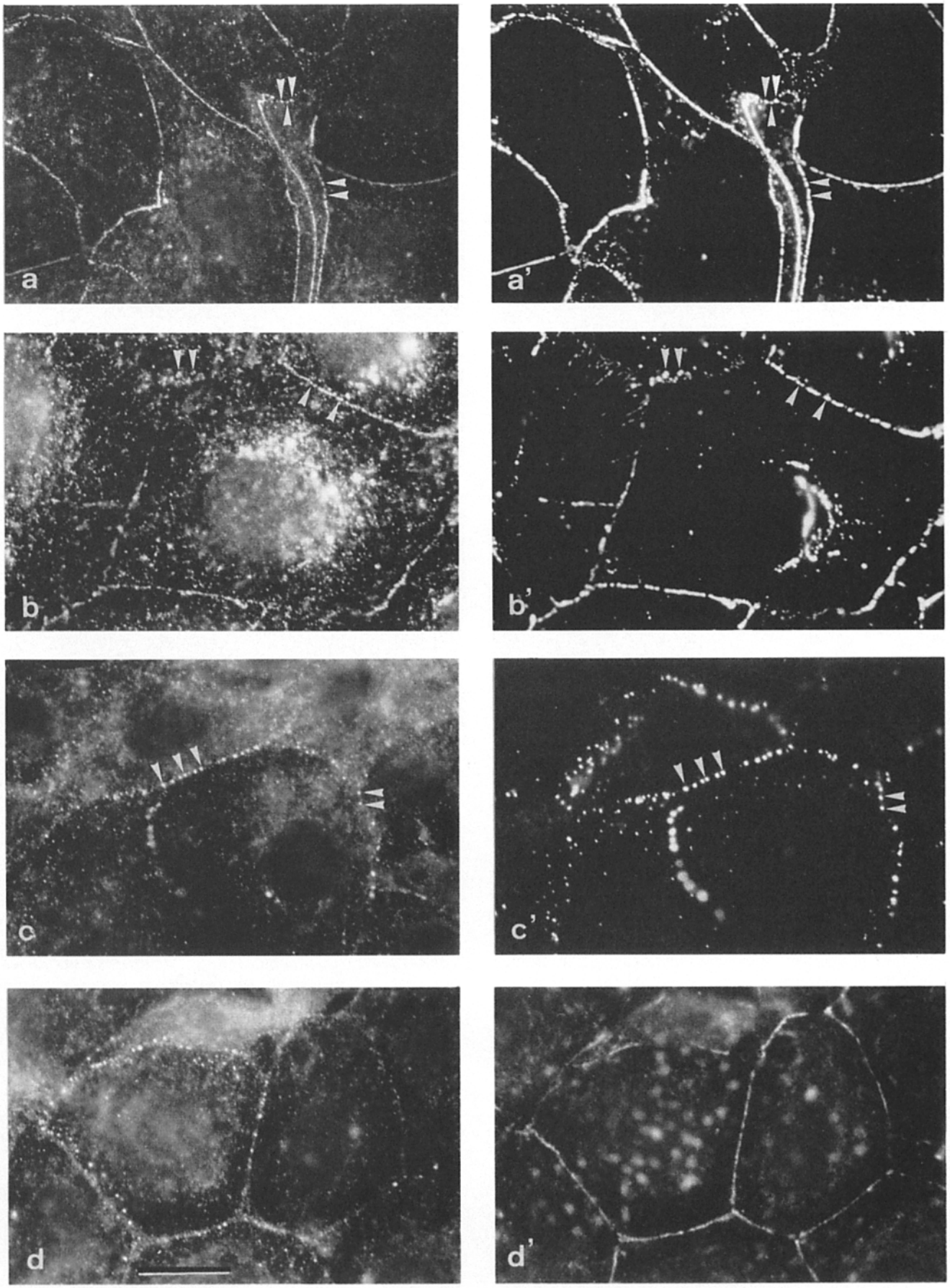
the rather homogeneous alignment of other microtubule-associated proteins, for example MAP4, along the length of microtubules (De Brabander et al., 1981). An accumulation of silver grains is also seen at regions of cell–cell contacts (Fig. 6 *b*) which can be clearly identified as desmosomes (Fig. 6 *c*). No labeling of intermediate filaments, which are very abundant in these regions, can be detected. The patches of pp170 aligned along microtubules (indicated by *arrows* in Fig. 6 *a*) may indicate association of this protein also with other structures. The fixation and embedding procedures used here do not, unfortunately, allow the investigation of possible associations of pp170 with membranous organelles, for example, because the cytoplasm and membranes are heavily extracted. Preliminary data suggest, however, that pp170 is also present on cytoplasmic membranes (Rickard, J. E., R. G. Parton, and T. E. Kreis, unpublished; see also Rickard and Kreis, 1990).

Desmosomes at the Cell Periphery Colocalize with Microtubules

Double staining and analysis of microtubules (Fig. 7, *a* and *b*) and pp170 (Fig. 7 *a'*) or desmosomes (Fig. 7 *b'*) in flat regions of subconfluent MDCK cells in the process of establishing cell–cell contacts, where these structures are well resolved, reveals a significant number of desmosomes that colocalize with microtubules (Fig. 7, *arrows*). Desmosomes are either aligned along microtubules or coincident with microtubule ends (Fig. 7, *b*,*b'*). Alignment of pp170 with microtubules and its association with microtubule ends was also demonstrated in MDCK cells (Fig. 7, *a*,*a'*). Clearly, further work including higher resolution electron microscopy and statistical analysis will be necessary to establish the significance of this close proximity of microtubules and desmosomes.

Effect of Microtubule-active Drugs on the Colocalization of pp170 with Desmosomes

It has been shown previously in HeLa cells that drugs which disrupt the normal microtubule network also lead to changes in the distribution of pp170. To test the effects of these drugs on the colocalization of pp170 with desmosomes, cells seeded at the same density but allowed to grow for either 1 d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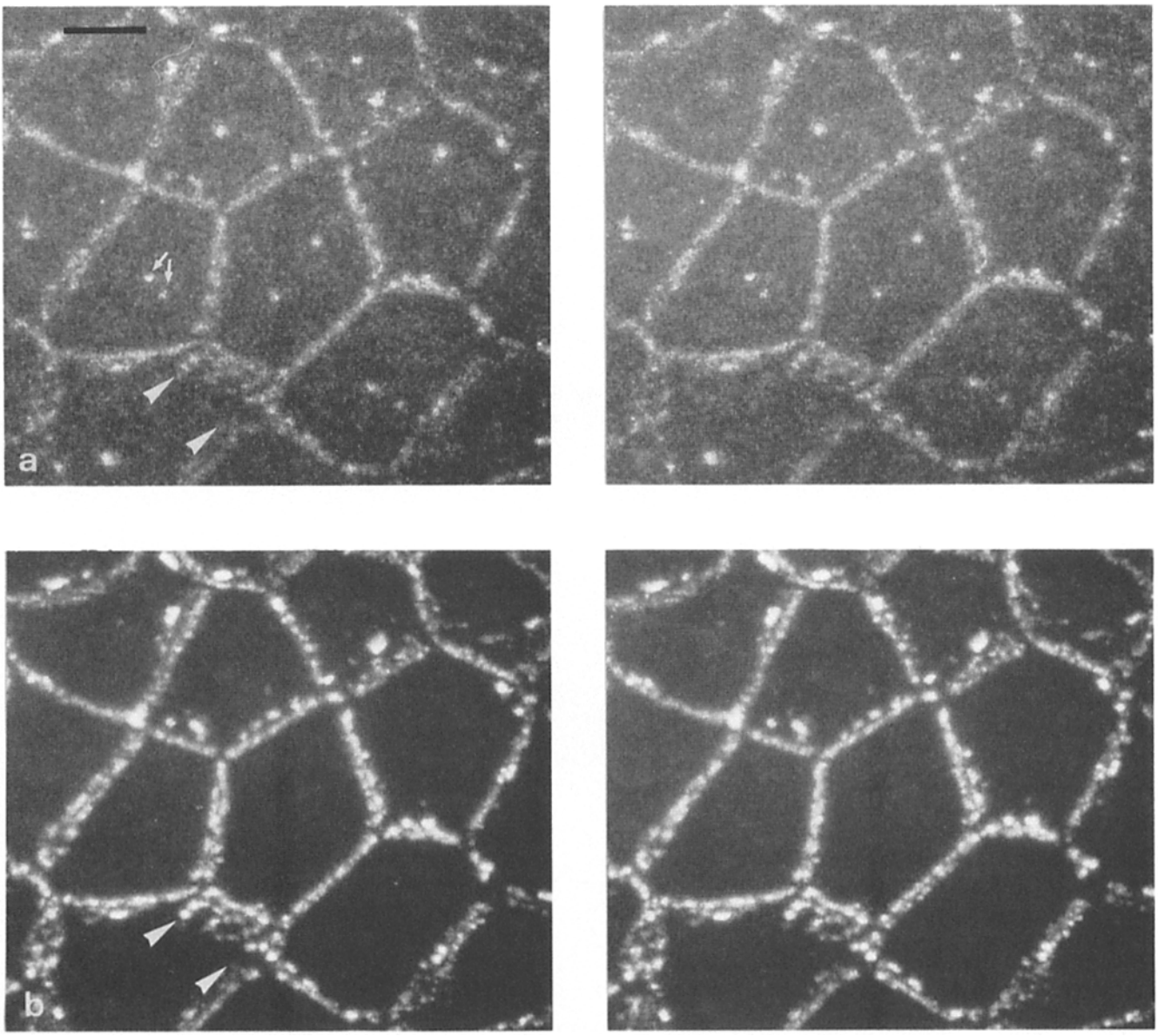


Figure 5. Colocalization of ppl70 and desmosomal protein by confocal microscopy in polarized MDCK cells. Polarized MDCK cells grown on filters were preextracted with detergent for 3 min before fixation in methanol/acetone. Cells were double labeled with rabbit α-ppl70 (*a*) and mouse antidesmosomal protein (*b*). Images of both fluorescence channels were recorded simultaneously with the confocal microscope using a vertical pitch of 1 μm. Stereo pairs were calculated for each channel separately using all sections from the series. Arrows indicate the position of centrosomes and arrowheads indicate an example of a region where both markers colocalize. For best viewing of the stereo pair pictures a mirror stereoscope (e.g., VCH; Stereo Optik, Dietzenbach) should be used. Bar, 5 μm.

to produce islands ("young cells"; Fig. 8, *c*, *e*, *g*) or 3 d to produce a very dense confluent monolayer ("old cells"; Fig. 8, *a*, *b*, *d*, *f*, *h*) were treated with 33 μM nocodazole, 10 μM vinblastine, or 10 μM taxol (Fig. 8). The effects of these drugs on the colocalization of ppl70 with desmosomes were evaluated by double immunolabeling after a 4-h incubation in medium containing each drug, a time period that leads to pronounced effects on the microtubule organization (Fig. 8, *c–h'*) when compared to untreated cells (Figs. 8 *a'* and 2 *c'*). For each of the three drugs tested we have found a difference

Figure 4. Colocalization of ppl70 and proteins of cell–cell junctions. MDCK (*a,a'*), Caco-2 (*b,b'*), and MDBK (*c,c'*, *d,d'*) cells were preextracted with detergent for 2 min (*a,a'*, *d,d'*) or 4x3 sec (*b,b'*, *c,c'*) before fixation with methanol/acetone. They were double labeled with α-ppl70 (*a–c*) and mouse antidesmosomal protein (*a'–c'*). Corresponding structures labeled by both antibodies, indicating colocalization of ppl70 with desmosomes, are indicated with arrowheads. Double labeling of MDBK cells with rabbit α-ppl70 (*d*) and mouse antivinculin antibodies (*d'*) show that ppl70 is not associated with the zonula adherens. The focus in (*d,d'*) is identical and in the plane of the adherens junction. Bar, 20 μm.

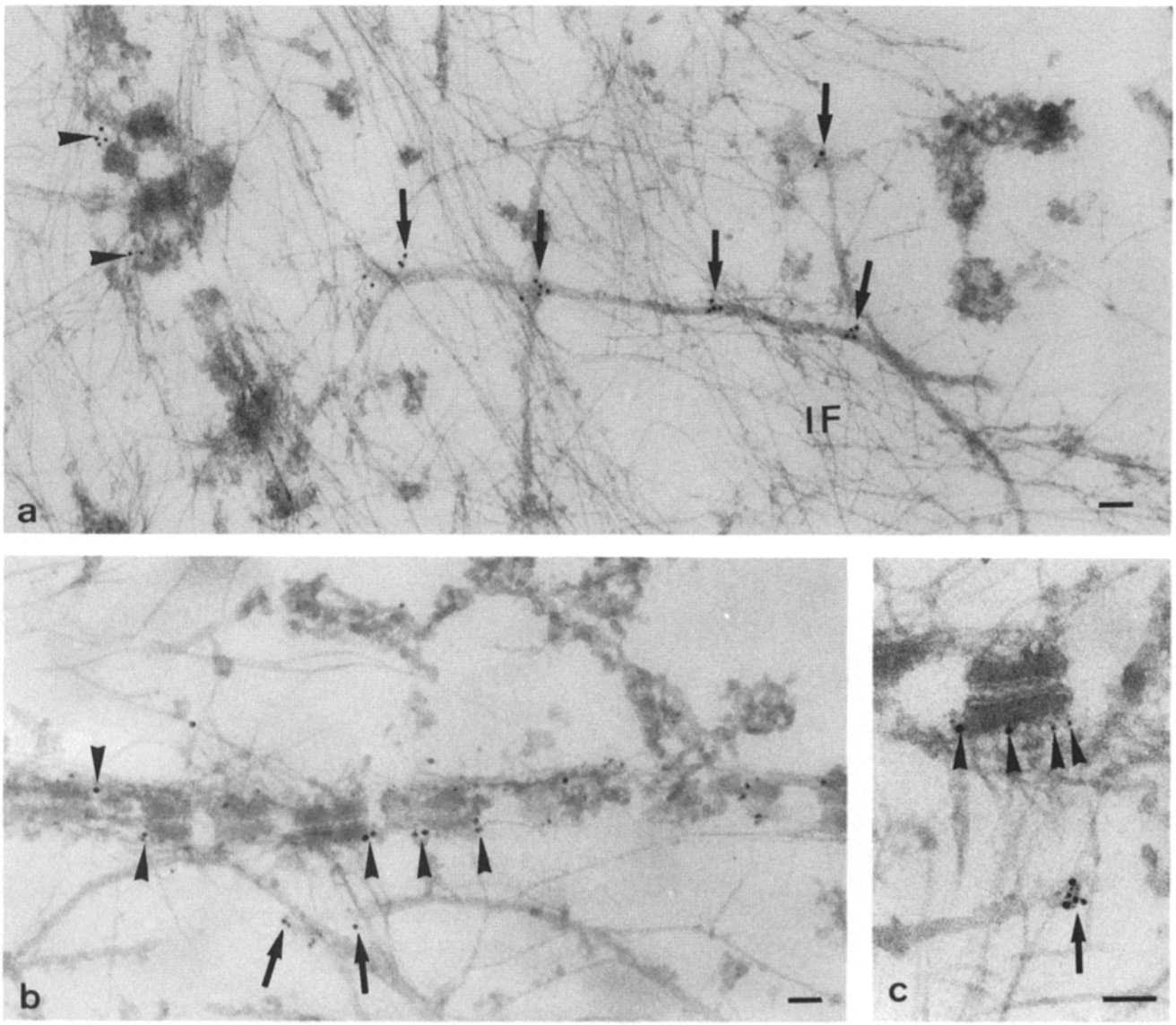


Figure 6. Immuno-electron microscopic localization of ppl70 at desmosomal plaques. Ultra-thin plastic sections of MDCK cells grown on glass coverslips were made after methanol/acetone fixation and preembedding labeling with α-ppl70 and 10-nm gold-conjugated (*a*) or 1-nm gold-conjugated secondary antibody, the latter visualized by silver enhancement (*b* and *c*). (*a*) Numerous gold particles are found along microtubules, often in distinct patches (*arrows*) and in regions of cell–cell contact (*arrowheads*). (*b* and *c*) Silver grains are localized at microtubules (*arrows*) and at desmosomal plaques (*arrowheads*). Intermediate filaments (*IF*) or other filamentous structures are not labeled. Bars, 0.1 μm.

in the association of ppl70 with desmosomes when the two types of cultures were compared.

Nocodazole, which completely depolymerizes the microtubule network (Fig. 8, *c'* and *d'*), leads in both cell populations to a pattern of ppl70 that is not diffuse but apparently associated with discrete cytoplasmic structures (Fig. 8, *c* and *d*), as has been shown previously for HeLa cells (Rickard and Kreis, 1990). Association of ppl70 with desmosomes, however, resists nocodazole treatment in young (Fig. 8 *c*) but not in old cells (Fig. 8 *d*). The desmosomes in old cells appear to remain unaffected by this treatment (Fig. 8 *b'*), but no ppl70 remains associated with desmosomes as shown in the corresponding picture of the same focal plane (Fig. 8 *b*). Thus, at the light microscopical level the organization of the desmosomes itself seems not to be affected either in young or old cells. Even after prolonged incubation of young cells with nocodazole (9 h) the amounts of ppl70 colocalizing with desmosomes remains comparable to control cells (not shown).

Treatment of MDCK cells with vinblastine leads to a reorganization of microtubules into paracrystals heavily labeled with α-ppl70. The vinblastine treatment also induces dissociation of ppl70 from the desmosomes of old (Fig. 8 *f*) but not young cells (Fig. 8 *e*).

Taxol treatment induces the formation of a dense microtubule network (Fig. 8, *g'* and *h'*), and in contrast to control cells, microtubules are absent from the regions where cells lie adjacent to each other, producing a nonlabeled rim around each cell (Fig. 8, *g'* and *h', arrows*) which is not seen in untreated cells (Figs. 2 *c'* and 8 *a'*). Surprisingly, ppl70

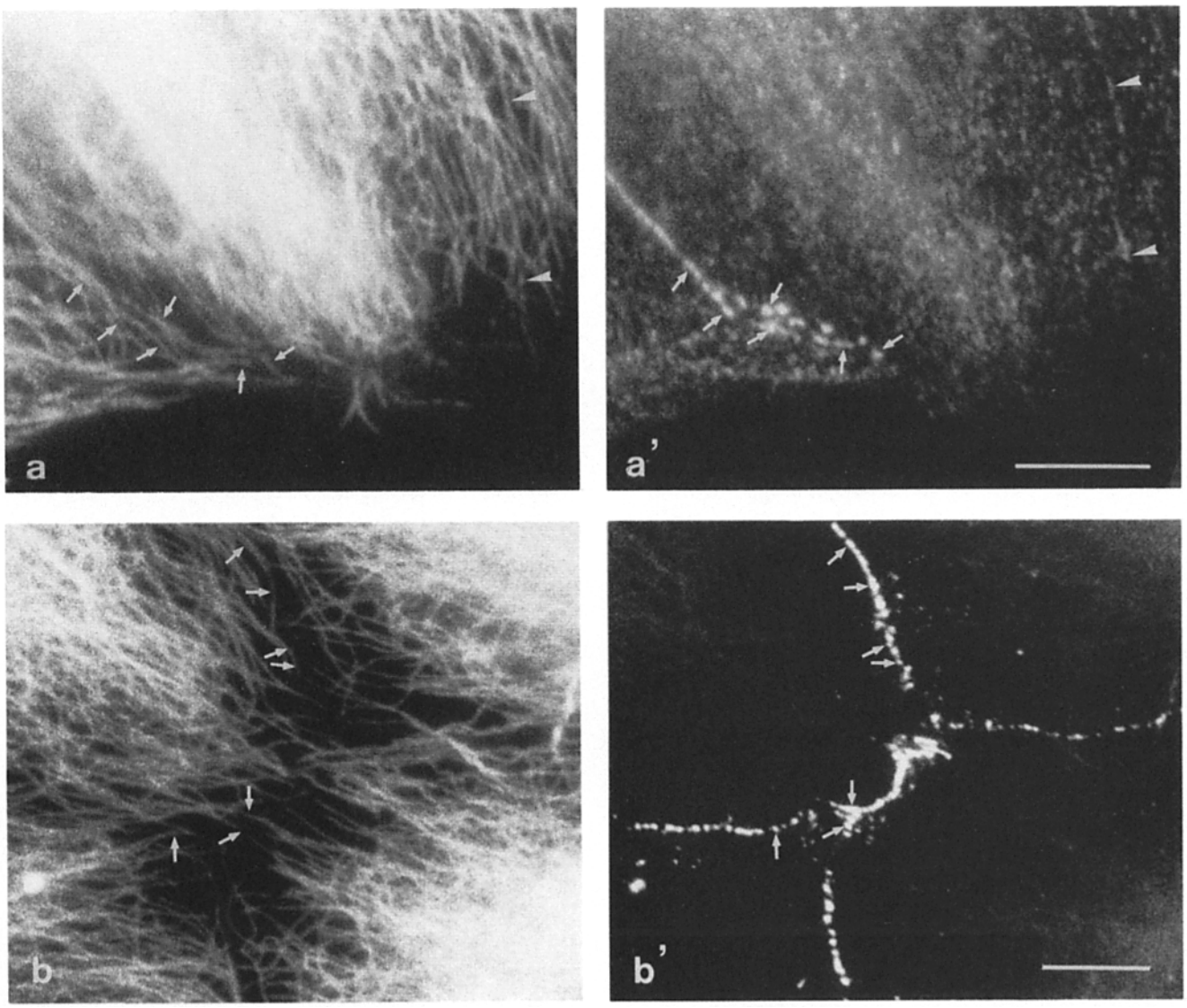


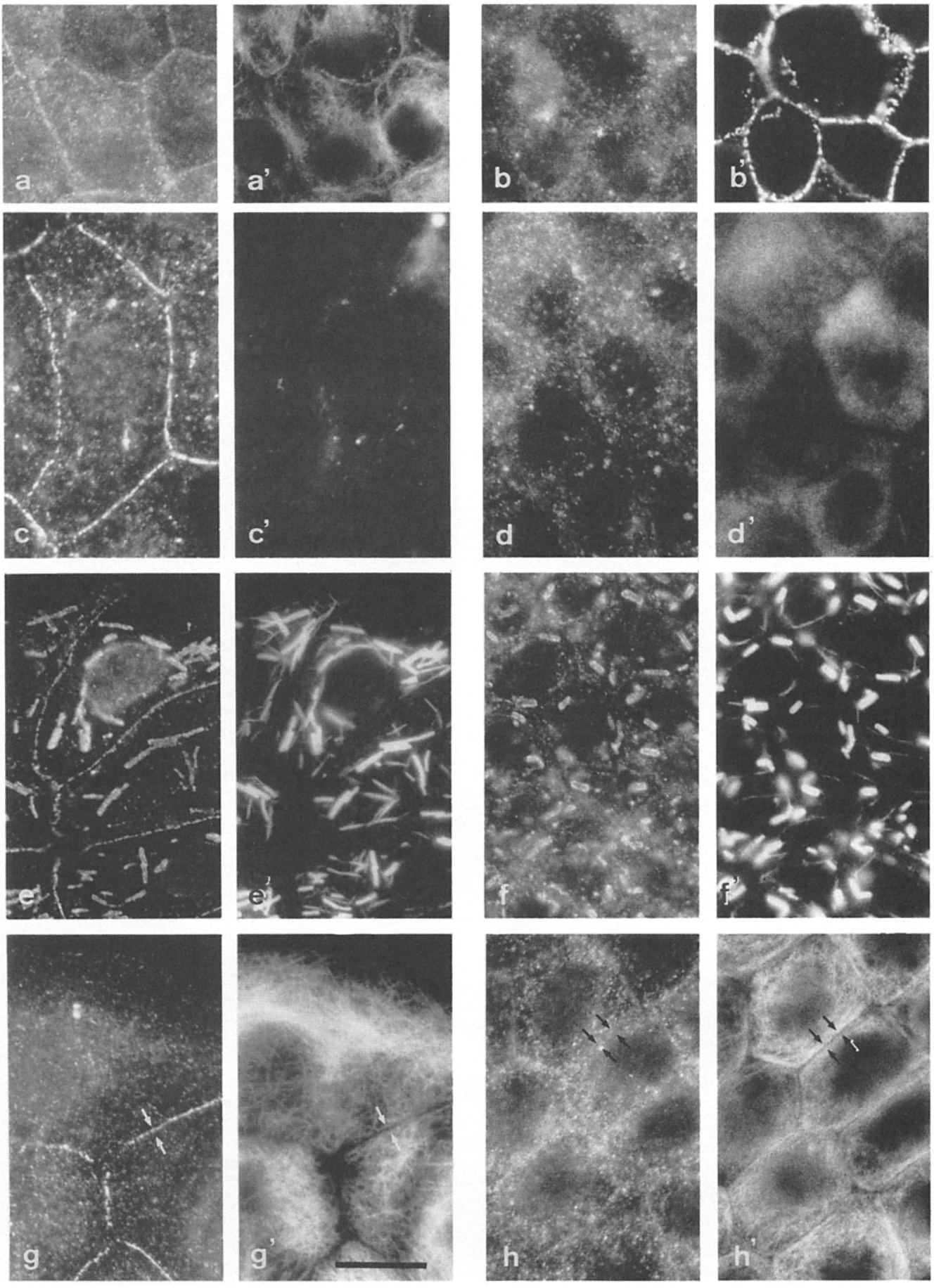
Figure 7. Colocalization of microtubules and desmosomes. Glass-grown MDCK cells were preextracted for 4 × 3 s, fixed with methanol/acetone as described in Materials and Methods. Cells were double labeled with mouse (*a*) or rabbit (*b*) antibodies against tubulin, and rabbit-α-ppl70 antibodies (*a'*) and mouse antibodies against desmosomal protein (*b'*). Arrowheads (*a,a'*) denote structures where ppl70 coincides with microtubules. Arrows indicate colocalization of ppl70 or desmosomal protein at "desmosomal patches" with microtubules. Bar, 10 μm.

also no longer colocalizes with desmosomes when old MDCK cells are treated with taxol for 4 h (Fig. 8 *h*), but does remain associated with desmosomes in young cells (Fig. 8 *g*), similar to what occurs when cells are treated with nocodazole or vinblastine. These effects of microtubule-active drugs suggest that the desmosome associated form of ppl70 in dense old populations of MDCK cells is in a labile state, in which it can dissociate from desmosomes after alteration of the integrity and the dynamic properties of the microtubule network, whereas in young sparse cultures, the association of ppl70 with desmosomes is more stable and apparently independent of microtubules.

Discussion

MDCK cells are an established model system for studying changes in microtubule dynamics and organization during the process of differentiation into a polarized epithelial monolayer (Pepperkok et al., 1990; Bré et al., 1990; Buendia et al., 1990; Bacallao et al., 1989). In this study we characterize the distribution of the microtubule binding protein ppl70 (Rickard and Kreis, 1990, 1991) in polarizing MDCK cells and find that a significant fraction of ppl70 accumulates at desmosomal plaques upon cell–cell contact formation.

The specificity of this colocalization of ppl70 with desmosomal plaques has been demonstrated by light microscopy using double immunofluorescence labeling and ultrastructurally, by immunoelectron microscopy. The following observations verify that the antibodies used in this study specifically react with ppl70, and do not crossreact with other proteins which localize at desmosomes as the cells establish cell–cell contacts. Firstly, the antibodies react specifically with ppl70 by immunoblotting. Secondly, ppl70 is found associated with microtubules as well as desmosomal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plaques by immunofluorescence and immunoelectron microscopy, and neither intermediate filaments nor other cytoskeletal structures are labeled with the antibodies. Thirdly, the association of ppl70 with desmosomes can be disrupted by treatment of MDCK cells with microtubule-active drugs. Furthermore, two antibodies (polyclonal α-ppl70 and monoclonal antibody 4D3), which recognize different epitopes on ppl70, label desmosomes.

Association of microtubules with specific domains at the plasma membrane has been shown in various cells, and it has been postulated that such an interaction may lead to stabilization of subsets of microtubules, reorganization of the cytoplasm, and morphogenesis (for a review see Kirschner and Mitchison, 1986). Rinnerthaler et al. (1988) have presented evidence for a role of microtubules in the definition of sites of cell-substrate interactions leading to the formation of more stable contact sites involving microfilament bundles. An association of microtubules (15 protofilaments) with specialized plasma membrane domains has also been demonstrated in epidermal cells of *Drosophila* wings, where transcellular bundles are anchored to desmosome-like structures (Mogensen et al., 1989) and in *Torpedo marmorata*, microtubules have been found associated with specialized domains of the postsynaptic membrane (Jasmin et al., 1991). The establishment of cell-cell contacts leads to the stabilization of microtubules in epithelial MDCK cells (Pepperkok et al., 1990; Bré et al., 1990), but not in fibroblasts (Pepperkok et al., 1990; Wadsworth et al., 1990). Binding of ppl70 to desmosomes in MDCK cells occurs concomitantly with the establishment of cellular junctions. This suggests that the protein may link microtubules to desmosomes and, thus, play an important role in triggering stabilization of microtubules. Further work is in progress to assess the occurrence of microtubule-desmosome interactions and to establish whether, indeed, an anchoring of microtubules via ppl70 to desmosomal plaques induces stabilization of microtubules.

It is well documented that intermediate filaments, mainly of the keratin, but also of the vimentin and desmin type, are associated with the cytoplasmic plaque of desmosomes (for reviews see Cowin et al., 1985; Jones and Green, 1991). To the best of our knowledge, anchoring or association of microtubules with genuine desmosomal plaques has not been reported before. The association of a microtubule-binding protein with desmosomes is, therefore, a novel and unexpected observation and raises the possibility of a functional interaction between desmosomes and microtubules. Interestingly, we find that "desmosomal patches", many of them not yet aligned in the belt of desmosomes (see Fig. 7), overlap with microtubules. Transport vesicles carrying desmoglein I, a membrane glycoprotein of the desmosomal membrane core domain, have been shown to colocalize with microtubule bundles after induction of cell contact in cells in which desmosome assembly was synchronized (Pasdar et al., 1991). Alternatively these patches may be immature desmosomes, and their coincidence with microtubules may thus suggest an important role for microtubules in the process of desmosome assembly and their spatial arrangement into a belt. Desmosomes may also interact with microtubules before their final alignment and play a role in the reorganization of the microtubule network at an early stage of cell-cell contact formation.

ppl70 dissociates from desmosomes upon depolymerization of microtubules only in old cells but not in young cells with perhaps less completely organized desmosomes. Differential sensitivity to desmosome disrupting activities, dependent on age and the differentiation state of MDCK cells, has been described in two reports. Desmosomes of MDCK cells become resistant to low Ca^{2+} after 4–5 d in normal growth medium (Mattey and Garrod, 1986), and treatment with a tumor promotor induces a rapid disruption of desmosomes in small colonies of MDCK cells, whereas in dense cultures the organization of desmosomal contacts is not affected (Ben-Ze'ev, 1986). The more stable association of ppl70 with the less organized desmosomes of young cells may suggest a more important role of this protein in the early establishment of cell-cell contacts and cell polarity.

So far nothing is known about the interaction partners of ppl70 at desmosomes. Desmosomes consist of at least eight proteins including the nonglycosylated desmosomal plaque proteins desmoplakins and plakoglobin, and the core membrane glycoproteins desmoglein and desmocollin (Kapprell et al., 1990; Schwarz et al., 1990; Garrod et al., 1990). Interestingly, significant sequence similarities between the extracellular domains of desmoglein and desmocollin and the corresponding domains of the Ca^{2+}-dependent cell adhesion molecules of the cadherin family suggests that desmosomes could also play a role in the cellular recognition process (Koch et al., 1990*a*,*b*; Collins et al., 1991; Mechanic et al., 1991; Nilles et al., 1991; Wheeler et al., 1991). The divergence in the sequence of the cytoplasmic domains of desmoglein, desmocollin, and cadherins presumably reflects their different interaction partners within the cell. An interaction of microtubules with desmosomes via ppl70 may occur directly with the cytoplasmic domain of one of these membrane proteins, with desmosomal plaque proteins or via other proteins at the periphery of desmosomes. Furthermore, the cell stage differential dissociation of ppl70 from cell-cell contact areas implies regulation of this association. Since ppl70 is a phosphoprotein (Rickard and Kreis, 1991) with a number of potential phosphoacceptor sites (Rickard, J. E., and T. E. Kreis, unpublished observations), it will be interesting to analyze whether phosphorylation regulates the interaction of ppl70 with desmosomes, and whether its phosphorylation state changes during cell differentiation. In vitro binding assays will be instrumental in defining the domains on ppl70 which are involved in its interaction with desmosomes, and they should allow identification of the cell-cell junction receptors for ppl70.

Figure 8. Effects of microtubule-active drugs on the distribution of ppl70. MDCK cells were grown on glass coverslips for 1 (*c*,*c'*, *e*,*e'*, *g*,*g'*) or 3 (*a*,*a'*, *b*,*b'*, *d*,*d'*, *f*,*f'*, *g*,*g'*) d after plating at equal density. Untreated cells (*a*,*a'*), or cells incubated for 4 h at 37°C with 33 μM nocodazole (*b*,*b'*, *c*,*c'*, *d*,*d'*), 10 μM vinblastine (*e*,*e'*, *f*,*f'*), or 10 μM taxol (*g*,*g'*, *h*,*h'*) were preextracted for 4 × 3 s with detergent before fixation with methanol/acetone. Fixed cells were doubled labeled with rabbit α-ppl70 antibodies (*a–h*), and mouse anti-tubulin (*a'*, *c'–h'*) or mouse antidesmosomal protein (*b'*) antibodies. Treatment with microtubule affecting drugs removes ppl70 from desmosomes of 3-d-old (*d*,*f*,*h*), but not 1-d-old (*c*,*e*,*g*) MDCK cells. Nocodazole has, however, no effect on the localization of desmosomal protein in three day old cells (*b'*). Corresponding regions are indicated by arrows. Bar, 20 μm.

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