

The Relationship between Intermediate Filaments and Microfilaments before and during the Formation of Desmosomes and Adherens-type Junctions in Mouse Epidermal Keratinocytes

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Abstract. Actin, keratin, vinculin and desmoplakin organization were studied in primary mouse keratinocytes before and during Ca^{2+} -induced cell contact formation. Double-label fluorescence shows that in cells cultured in low Ca^{2+} medium, keratin-containing intermediate filament bundles (IFB) and desmoplakin-containing spots are both concentrated towards the cell center in a region bounded by a series of concentric microfilament bundles (MFB). Within 5–30 min after raising Ca^{2+} levels, a discontinuous actin/vinculin-rich, submembranous zone of fluorescence appears at cell–cell interfaces. This zone is usually associated with short, perpendicular MFB, which become wider and longer with time. Later, IFB and the desmoplakin spots are seen aligned along the perpendicular MFB as they become redistributed to cell–cell interfaces where desmosomes form.

Ultrastructural analysis confirms that before the Ca^{2+} switch, IFB and desmosomal components are found predominantly within the perimeter defined by the out-

ermost of the concentric MFB. Individual IF often splay out, becoming interwoven into these MFB in the region of cell–substrate contact. In the first 30 min after the Ca^{2+} switch, areas of submembranous dense material (identified as adherens junctions), which are associated with the perpendicular MFB, can be seen at newly formed cell–cell contact sites. By 1–2 h, IFB–desmosomal component complexes are aligned with the perpendicular MFB as the complexes become redistributed to cell–cell interfaces. Cytochalasin D treatment causes the redistribution of actin into numerous patches; keratin-containing IFB undergo a concomitant redistribution, forming foci that coincide with the actin-containing aggregates. These results are consistent with an IF–MF association before and during desmosome formation in the primary mouse epidermal keratinocyte culture system, and with the temporal and spatial coordination of desmosome and adherens junction formation.

CURRENT evidence suggests that the major cytoskeletal elements, microtubules, microfilaments (MF),¹ and intermediate filaments (IF), interact within cultured cells to form complex and perhaps functionally important arrays. Associations between microtubules and IF and between microtubules and MF have been reported (e.g. 2, 14, 17, 19, 34, 39, 40). Relatively few investigations have focused on a possible relationship between IF and MF. Several reports have mentioned the possibility of an association between IF and MF in or near the dense bodies of smooth muscle cells (4, 43, 44), and one report discussed the copurification of actin and IF protein from smooth muscle (23). Others have

reported possible MF–IF interactions in intestinal brush border (22, 24), cultured fibroblasts (18, 41), and liver (38). Recently, keratin-containing intermediate filament bundles (IFB) have been shown to become redistributed upon perturbation of the MF system in certain epithelial cells and appear to be colocalized with resulting actin-containing structures (3, 52).

In this paper we show that cultured keratinocytes provide an excellent opportunity to study relationships between IF and MF. In cultured kidney epithelial cells, human keratinocytes, and the primary mouse epidermal (PME) cell system used here, the organization of the IF cytoskeleton and formation of desmosomes at cell–cell interfaces can be manipulated by changing exogenous levels of Ca^{2+} (20, 21, 27, 35, 51). Jones and Goldman (27) have provided evidence that in PME cells, IFB and preformed desmosomal components

1. *Abbreviations used in this paper:* CD, cytochalasin D; IF, intermediate filaments; IFB, intermediate filament bundles; LCa^{2+} , low Ca^{2+} medium; MF, microfilaments; MFB, microfilament bundles; NCA^{2+} , normal Ca^{2+} medium; PME, primary mouse epidermal.

(i.e., desmoplakin 1-containing structures) are associated in the cytoplasm, and that raising the Ca^{2+} levels (from ~ 0.1 to 1.2 mM) induces their concomitant redistribution toward the cell-cell interfaces where mature desmosomes are formed.

Here, we demonstrate that before and after the Ca^{2+} switch, even in the presence of cytochalasin D (CD), IFB maintain specific structural relationships with the MF system. Furthermore, we show that movement of the desmosome component-IFB complexes to the cell margins is related in a temporal and spatial manner with MF-associated adherens junction formation. The latter type of junction (which includes the *zonula adherens* of polarized epithelia, focal contacts of cultured cells, dense plaques of smooth muscle, and *fascia adherens* of cardiac muscle [10, 11, 13]) is identified using criteria established by Geiger and his colleagues, one of which is the specific association of adherens junction membrane domains with vinculin (8, 13, 15, 16, 47).

Our studies suggest that the PME culture system provides a useful model for studying IF-MF interactions and the relationship of these cytoskeletal elements to the formation of two types of junction, i.e., the desmosome and the adherens junction, both of which are thought to play important roles in cellular morphogenesis and the mediation of cell-cell interactions (25, 33, 36, 37, 46).

Materials and Methods

Cell Culture

PME cells were prepared by the trypsin flotation procedure of Yuspa and Harris (53). They were maintained in low Ca^{2+} medium (LCa^{2+}) as described by Jones and Goldman (27). The developmental program of cytoskeletal reorganization and junction formation was induced by removing the LCa^{2+} and replacing it with Eagle's minimum essential medium containing the normal concentration of Ca^{2+} plus 10% fetal calf serum, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (normal Ca^{2+} medium, NCa^{2+}).

Drug Treatment

CD was made up as a 1-mg/ml stock in DMSO and diluted to a concentration of 0.5 $\mu\text{g}/\text{ml}$ in culture medium. For immunofluorescence experiments, cells growing at low density on coverslips were exposed to CD in LCa^{2+} for 2 h before switching. Cells were then switched to NCa^{2+} containing CD and fixed at timed intervals. For electron microscopy (EM) cells growing at high density on plastic dishes were exposed to the same CD regimen and fixed at timed intervals (see fixation protocols below). Cells incubated in medium containing DMSO but not CD appeared identical to cells maintained in normal culture medium.

Probes Used for Fluorescence Microscopy

Two monoclonal antibodies were used for immunofluorescence: a previously described rat monoclonal anti-keratin (29) and a mouse monoclonal anti-vinculin (16, 47). In addition, a rabbit antiserum directed against desmoplakin 1 from bovine muzzle desmosomes (which recognizes both desmoplakin 1 and 2, references 1 and 27) was used. Rhodamine-phalloidin (Molecular Probes Inc., Junction City, OR) was used as a fluorescent labeling probe for actin. Secondary antibodies used included fluorescein-conjugated goat anti-rabbit IgG (Nordic Immunological Laboratories, El Toro, CA), fluorescein-conjugated goat anti-rat IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD), fluorescein- or rhodamine-conjugated goat or rabbit anti-mouse IgG (Miles-Yeda, Rehovot, Israel).

Fluorescence Microscopy

PME cells growing on coverslips were fixed for 30 s–5 min in 3.7% formaldehyde in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline (PBSa: 6 mM phos-

phate, 171 mM NaCl, 3 mM KCl, pH 7.4), rinsed in distilled water, and subsequently extracted in ice-cold acetone for 2 min. When vinculin plus actin were to be visualized the longer fixation time of 5 min was used. For double-label fluorescence using actin or vinculin with either keratin or desmoplakin, shorter incubations in formaldehyde were used. After acetone extraction, further steps were carried out as described elsewhere (18, 28). Both sequential and simultaneous incubations were performed without any difference in resulting patterns. Single-label fluorescence controls were performed without resulting in any change in patterns as compared with double-label analysis.

Combinations of primary probes used were: rhodamine-phalloidin (diluted 1:30 in PBSa) plus anti-keratin (final dilution 1:1 of hybridoma medium), rhodamine-phalloidin plus anti-vinculin (undiluted hybridoma medium); rhodamine-phalloidin plus anti-desmoplakin (diluted 1:50). Double-label analysis of desmoplakin and keratin previously described (27) was performed here only as a control.

A Zeiss Photomicroscope III equipped with epifluorescence optics was used for immunofluorescence and phase-contrast observations and photography as described previously.

EM and Decoration with Myosin Subfragment-1 (S-1)

Cells in petri dishes were fixed (27), dehydrated, and embedded (45) as previously described. For in situ decoration experiments, myosin S-1 was prepared and used under conditions described previously (42). Thin sections were made on a Nova Microtome (LKB Instruments, Inc., Gaithersburg, MD) using a diamond knife, mounted on uncoated copper grids, and stained with uranyl acetate followed by lead citrate. Transverse sections were prepared by polymerizing portions of two flat embedded cell monolayers together with Epon-Araldite (Electron Microscopy Sciences, Fort Washington, PA) and by sectioning the resulting block perpendicular to the growth substrate. Thin sections were examined with either a JEOL 100CX or JEOL 200CX electron microscope.

Results

Fluorescence Microscopy

In the presence of LCa^{2+} , keratin-containing IFB of PME cells are concentrated toward the cell center; IFB of neighboring cells do not associate at cell-cell contact areas (27). Double-label fluorescence analysis using rhodamine-phalloidin and the monoclonal anti-keratin antibody was carried out. The actin-containing cytoskeleton visualized by this procedure appears to depend somewhat on cell shape. Most frequently it is composed of numerous concentrically arranged microfilament bundles (MFB).² These MFB are usually circular or polygonal in shape (Fig. 1, *a* and *c*); however, occasionally the MFB create only partial circles or polygons (e.g., Fig. 1 *e*). The concentric MFB are intersected by a series of radial, spokelike MFB that extend from the cell center to the cell margins (Fig. 1, *a*, *c*, and *e*). In addition, in some cells more diffuse regions of actin fluorescence are also seen (Fig. 1 *a*).

IFB are restricted to the boundaries defined by the outermost of the concentric MFB, only occasionally extending into the cell margins (Fig. 1, *a* and *b*). Double-label fluorescence using rhodamine-phalloidin and anti-desmoplakin reveals that desmoplakin-containing spots are also concentrated within the region defined by the outermost of the concentric MFB (Fig. 1, *c* and *d*). In PME cells maintained in LCa^{2+} medium, the anti-vinculin monoclonal antibody reacts with focal contact-like patches that are co-distributed

2. Although strictly defined "microfilament bundle (MFB)" is an ultrastructural term, for clarity we use this phrase throughout the Results section. The MF-containing nature of these light microscopic fibers is confirmed in the ultrastructural analysis described below.

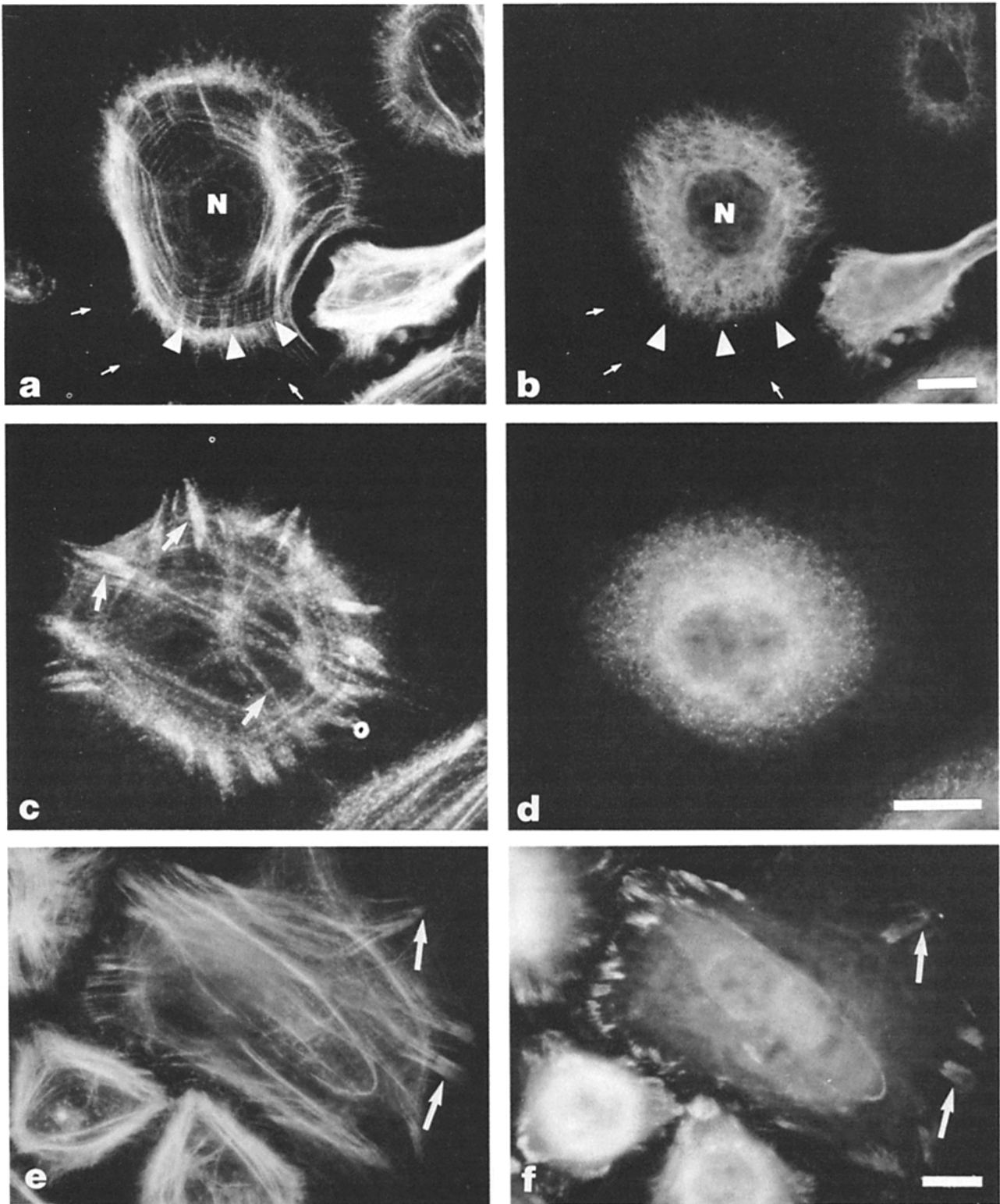


Figure 1. Double-label immunofluorescence micrographs of PME cells in LCa^{2+} . (a) Actin pattern (visualized by incubating with rhodamine-phalloidin) and (b) keratin (IFB) pattern (incubated with monoclonal anti-keratin followed by anti-rat fluorescein) in the same cell. Note that IFB are distributed between the nucleus (N) and the outermost circular MFB (large arrowheads). The cell periphery is marked by small arrows. (c) Actin (rhodamine-phalloidin) and (d) desmoplakin (rabbit anti-desmoplakin) patterns in the same cell. Desmoplakin staining is concentrated within the outermost circular MFB. Radial MFB are denoted by arrows. (e) Actin (rhodamine-phalloidin) and (f) vinculin (mouse monoclonal anti-vinculin) patterns. Vinculin staining is found primarily at the termini of radial MFB (arrows). Bars: (a-e) 10 μ m.

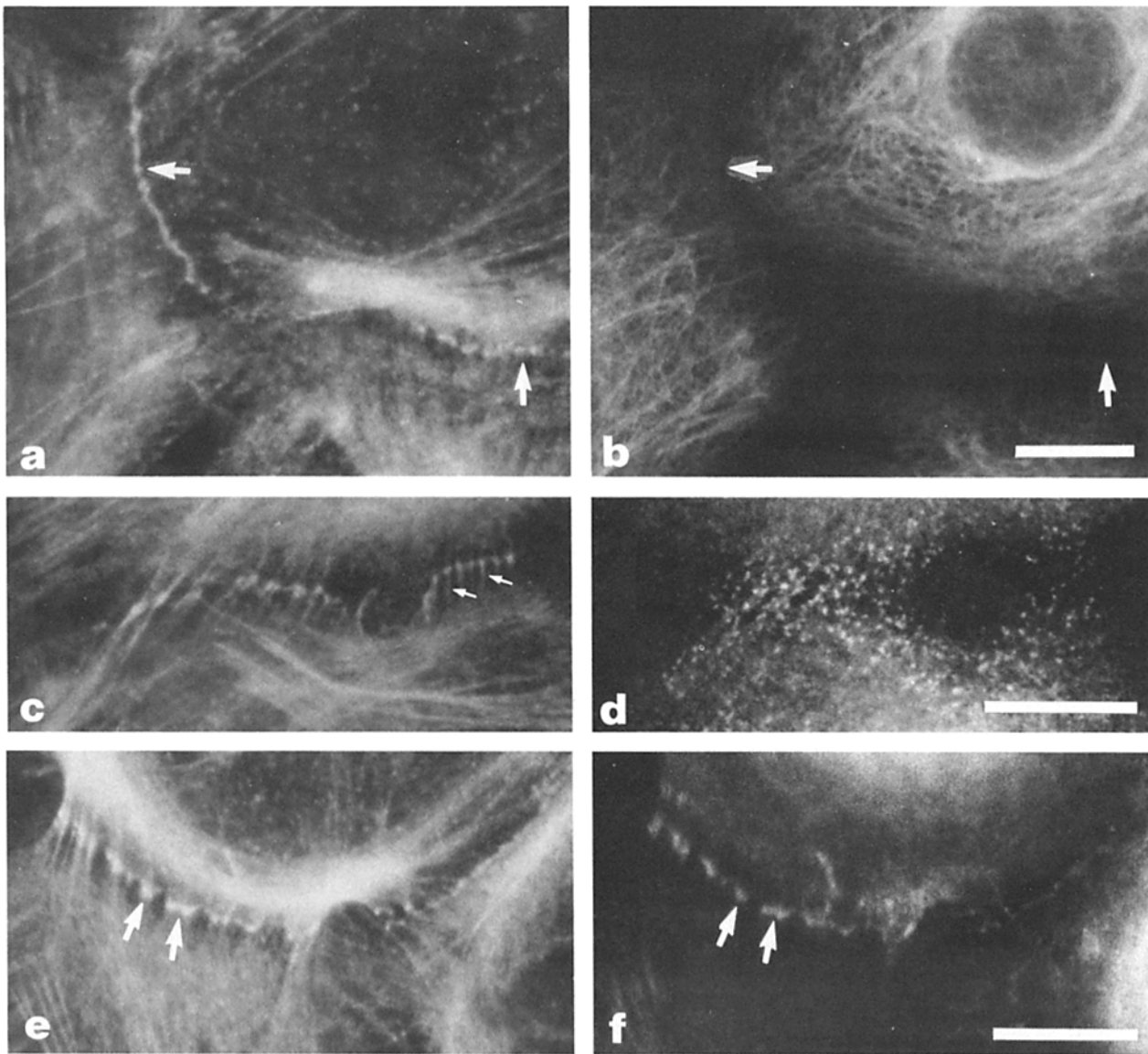


Figure 2. Double-label immunofluorescence micrographs of PME cells 30–45 min after switch to NCa^{2+} . (a) Actin (rhodamine-phalloidin) and (b) keratin patterns in the same cell. Note that IFB are not yet associated with the cell surface (cf. Fig. 3 b) even though the submembranous zone of actin has formed (arrows). (c) Actin and (d) desmoplakin patterns in the same cell. Desmoplakin-containing spots are distributed throughout the cell–cell contact zone (indicating desmosomes are not yet formed) even though the discontinuous actin zone has formed. Short, perpendicular MFB are marked with arrows. (Compare this early distribution of desmoplakin with Fig. 3 a in reference 27). (e) Actin and (f) vinculin patterns. Vinculin staining corresponds precisely with the discontinuous actin-containing zone (arrows). Bars: (a–e) 10 μm .

with the distal ends of the radial MFB, at the cell–substratum interface (Fig. 1, e and f).

After raising Ca^{2+} levels to 1.2 mM (NCa^{2+}), conspicuous changes in all four patterns (keratin, actin, desmoplakin, and vinculin) are observed. Between 5 and 30 min after the switch, phalloidin staining reveals a brightly fluorescent submembranous zone in regions of cell–cell contact (Fig. 2, a, c, and e). This zone is often discontinuous in nature. (As described below in the EM section, this zone is probably equivalent to the electron-dense plaques characteristic of intercellular adherens junctions). Associated with this submembranous actin are short MFB that stain less intensely and are oriented perpendicular to the cell–cell contact zone (Fig. 2, c and e). These MFB increase in length and width

with time. No apparent change is seen in the concentric MFB or in the focal contact–associated MFB.

Jones and Goldman (27) have shown that IFB also become associated with the surface of PME cells shortly after raising Ca^{2+} levels. We studied the keratin and actin patterns simultaneously by double-label immunofluorescence within 30 min after the switch. Micrographs show that in many cells very few IFB are associated with the cell surface, even though the brightly fluorescent actin zone with associated MFB is already present at cell–cell interfaces (Fig. 2, a and b). When anti-desmoplakin and rhodamine-phalloidin are used simultaneously, it is clear that, in many cells, at 30 min after the switch to NCa^{2+} the submembranous actin zone with associated MFB has already developed, whereas desmoplakin-

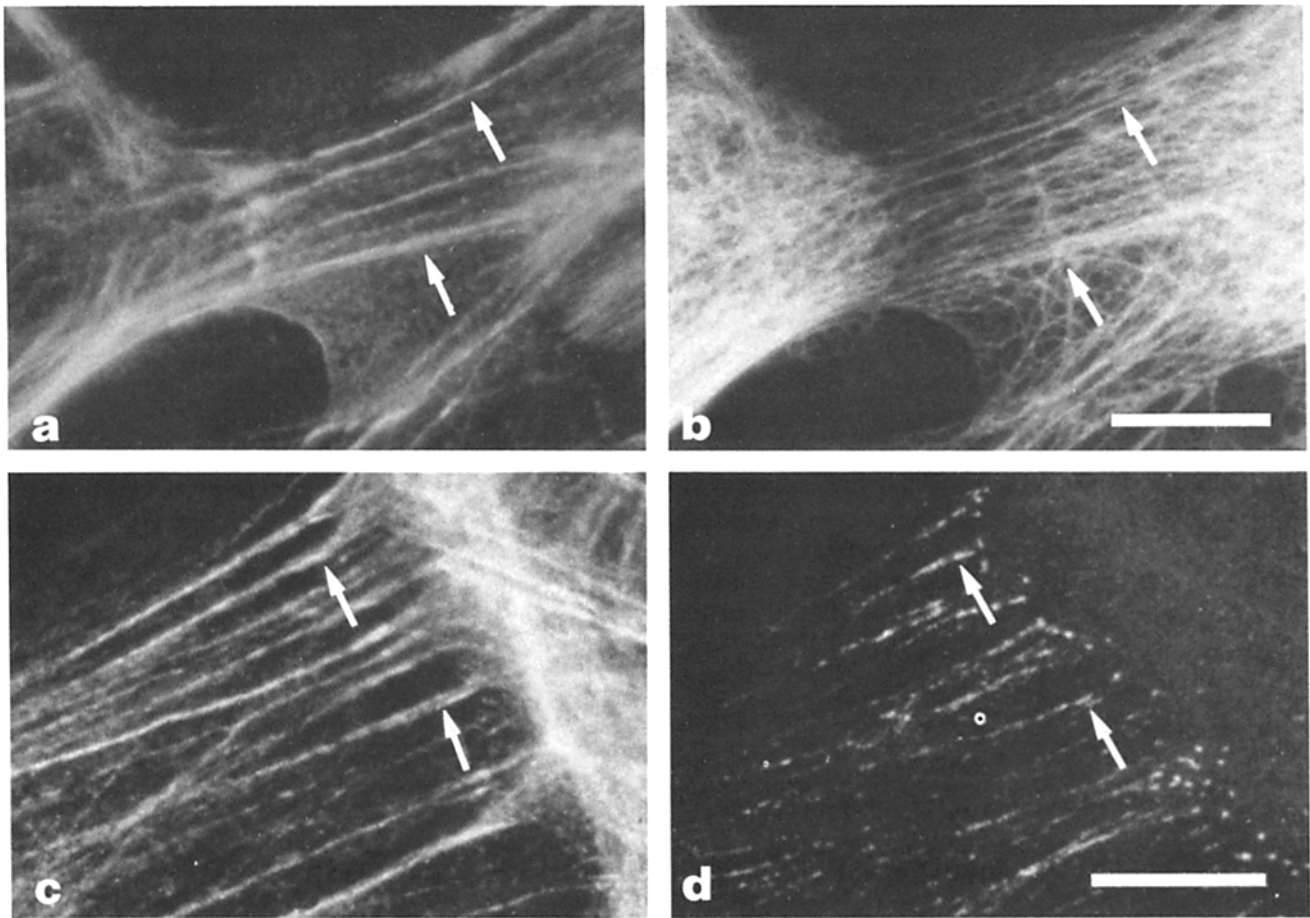


Figure 3. Double-label fluorescence of PME cells more than 3 h after Ca^{2+} switch. (a) Actin and (b) keratin patterns in the same cell show parallel alignment of IFB and MFB (arrows). (c) Actin and (d) desmoplakin distribution in the same cell. In this cell, desmosome formation began fairly late after the switch, after actin bundles had developed extensively. Alignment of desmoplakin spots with MFB is therefore quite dramatic (arrows). Bars: (a–d) 10 μm .

staining spots remain distributed throughout the peripheral cytoplasm (Fig. 2, c and d). These data are consistent with the later time course for IFB redistribution. During the Ca^{2+} -dependent reorganization, vinculin staining corresponds precisely with phalloidin staining in the discontinuous fluorescent zone at cell–cell interfaces (Fig. 2, e and f). In addition, patches of actin/vinculin co-staining at focal contacts are still seen. Double-label fluorescence analysis of vinculin and desmoplakin distribution during these early stages after the Ca^{2+} switch confirm that the vinculin-containing fluorescent zone at cell–cell interfaces develops before the final stages of desmoplakin reorganization (data not shown).

As time proceeds, IFB and desmoplakin spots become redistributed to cell–cell contact sites (27). By 1–3 h after the Ca^{2+} switch, many of these IFB and MFB appear to run parallel to one another. These arrays are generally oriented perpendicular to cell–cell contact areas (Fig. 3, a and b). Linear arrays of desmoplakin spots also often appear to coincide with MFB (Fig. 3, c and d). Subsequently, desmosomes form along cell–cell interfaces, often resulting in brightly fluorescent lines of varying lengths (reference 27, Figs. 3 a and 4 c). It is important to note that along cell borders where there are no neighboring cells there appears to be no redistribution of these cytoskeletal components.

Immunofluorescence results suggest, therefore, a temporal sequence of events that occurs after the switch to NCa^{2+} . First, a discontinuous actin/vinculin-rich, submembranous zone, in association with short MFB, appears in regions of cell–cell contact. Desmoplakin-containing spots and IFB become redistributed with a later time course; frequently these elements are aligned with MFB during the reorganization. Eventually desmoplakin-containing spots become localized at cell–cell interfaces where desmosomes are formed (27). The time course with which these events are observed varies within a cell population. It is important to note, however, that the relative timing between the formation of actin/vinculin-rich cell–cell contacts and desmoplakin/IFB redistribution at individual cell–cell interfaces apparently remains constant, i.e., actin/vinculin contacts form before desmosomes.

EM

Ultrastructural analyses of conventionally fixed, intact PME cells as well as permeabilized cells incubated in myosin S-1 reveal, in more detail, the relationship between MF, IF, and associated junctions during cell contact formation.

Figs. 4 and 5 a confirm the light microscope observations that in LCa^{2+} the keratin-containing cytoskeletal system of PME cells is concentrated toward the cell center and for the most part is confined within the outermost concentric MFB.

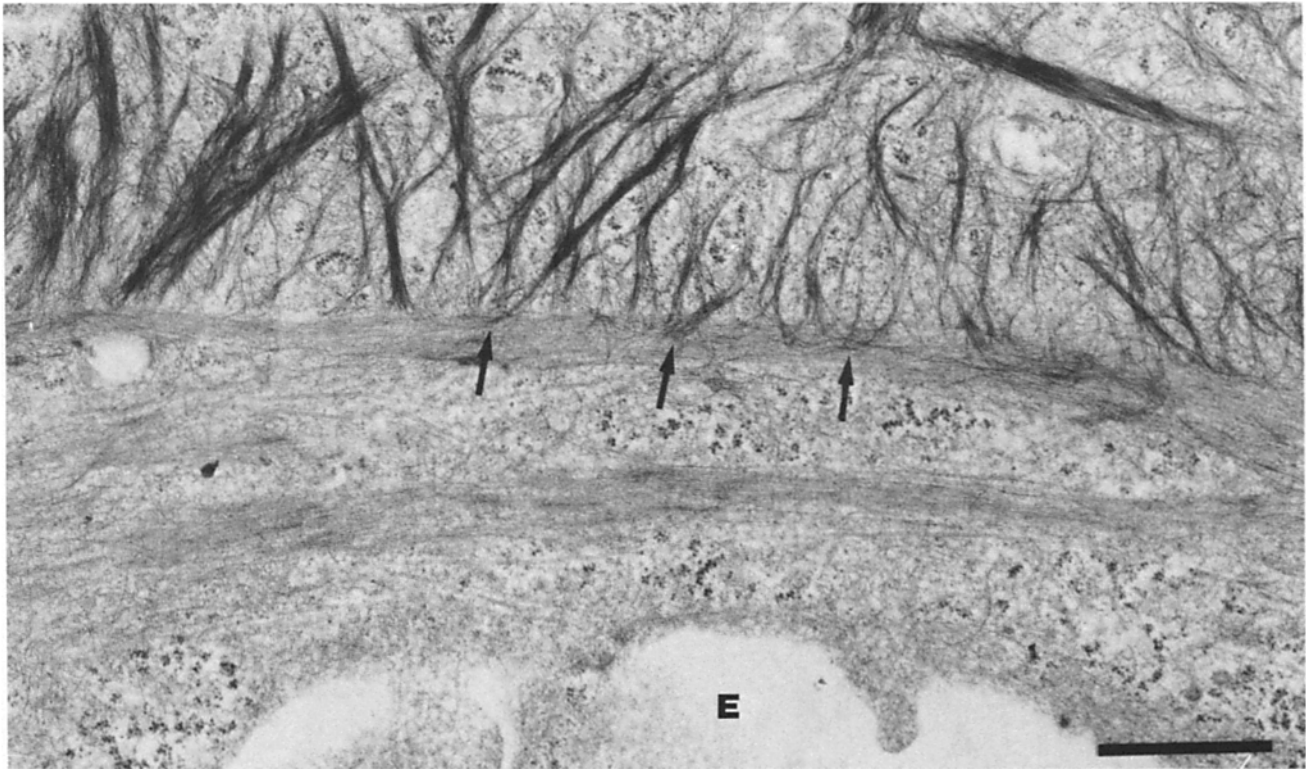


Figure 4. EM of unswitched cell. IFB appear to splay out at the juncture of IF and the circular MFB and become interwoven with or loop through MF comprising the bundle (arrows). Extracellular space is marked E. Bar, 1 μ m.

At the juncture of the IFB and MFB, IFB splay out and individual IF appear to become interwoven with MF comprising the bundle (Fig. 4, arrows, and Fig. 5 a). Furthermore, IFB can often be seen in close association with MF (identified by decoration with myosin S-1) composing the radial bundles seen in immunofluorescence micrographs (Fig. 5, a and b).

Analysis of transverse sections confirms that bundles of IFB and MFB are closely associated in PME cells maintained in LCa^{2+} , and provides additional information regarding the three-dimensional organization of the two filament systems. In sections containing the nucleus, the IFB "cage" that surrounds the nucleus (see reference 28) appears to be continuous with a cytoplasmic network. In the cortical cytoplasm adjacent to the cell-substrate adhesion zone, IFB appear to terminate near the cell margins at the level of MFB (Fig. 6 a). On either side of the nucleus, cross-sectional profiles of the two filament systems are frequently seen in close association at a single level between the dorsal and ventral surfaces of the cell (Fig. 6 b).

Ultrastructural analysis of unswitched cells also confirms the perinuclear location of IFB and closely associated electron-dense bodies (Fig. 7, arrows). Few of these complexes are seen in the cell margins. Jones and Goldman (27) have identified these bodies as desmoplakin-containing complexes. The complexes are probably equivalent to the desmoplakin-containing spots seen in immunofluorescence micrographs and apparently migrate toward cell-cell interfaces where they are involved in desmosome formation.

After the switch to NCa^{2+} , IFB retain their association with radial MFB and the concentric MFB, and, in addition,

appear to be related in the junction forming region. Within the first half hour after the switch, dense, submembranous plaques are visible at intercellular contact sites (Fig. 8 a). The plaques are associated with MFB oriented perpendicular to the cell margins. These MF-associated submembranous plaques appear to be very similar to intercellular adherens junctions (AJ in Fig. 8 a) described by others, (e.g., 6, 11, 13, 24) and presumably represent the actin/vinculin-rich submembranous zones seen in immunofluorescence micrographs. Mature desmosomes are seldom present. However, in many cells IFB-desmoplakin complexes frequently appear to line up adjacent to the MFB associated with adherens junctions (Fig. 8, a and b). Even after desmosomes are formed, they and their associated IF maintain a close association with adjacent adherens junctions (Fig. 8 c). During and after desmosome formation, IFB also appear to be interwoven through the peripheral circular MFB that earlier defined the outermost boundaries of the keratin network (Fig. 8 d). Some of these IFB appear to course from the nuclear region to the circular MFB, just as before the Ca^{2+} switch (see Fig. 1). Other IFB extend from the MFB toward the cell margins; their termini are associated with electron-dense bodies or fully formed desmosomes (Fig. 8 d).

Examination of cross sections throughout the process of junction formation reveals that desmosomes and adherens junctions form at cell-cell interfaces at the level of the MF-IF network that is present before the Ca^{2+} switch. Adherens junctions in these cells are found in positions not only ventral, but also dorsal, to desmosomes (data not shown). From 3 to 6 h after the Ca^{2+} switch, observations of oblique sec-

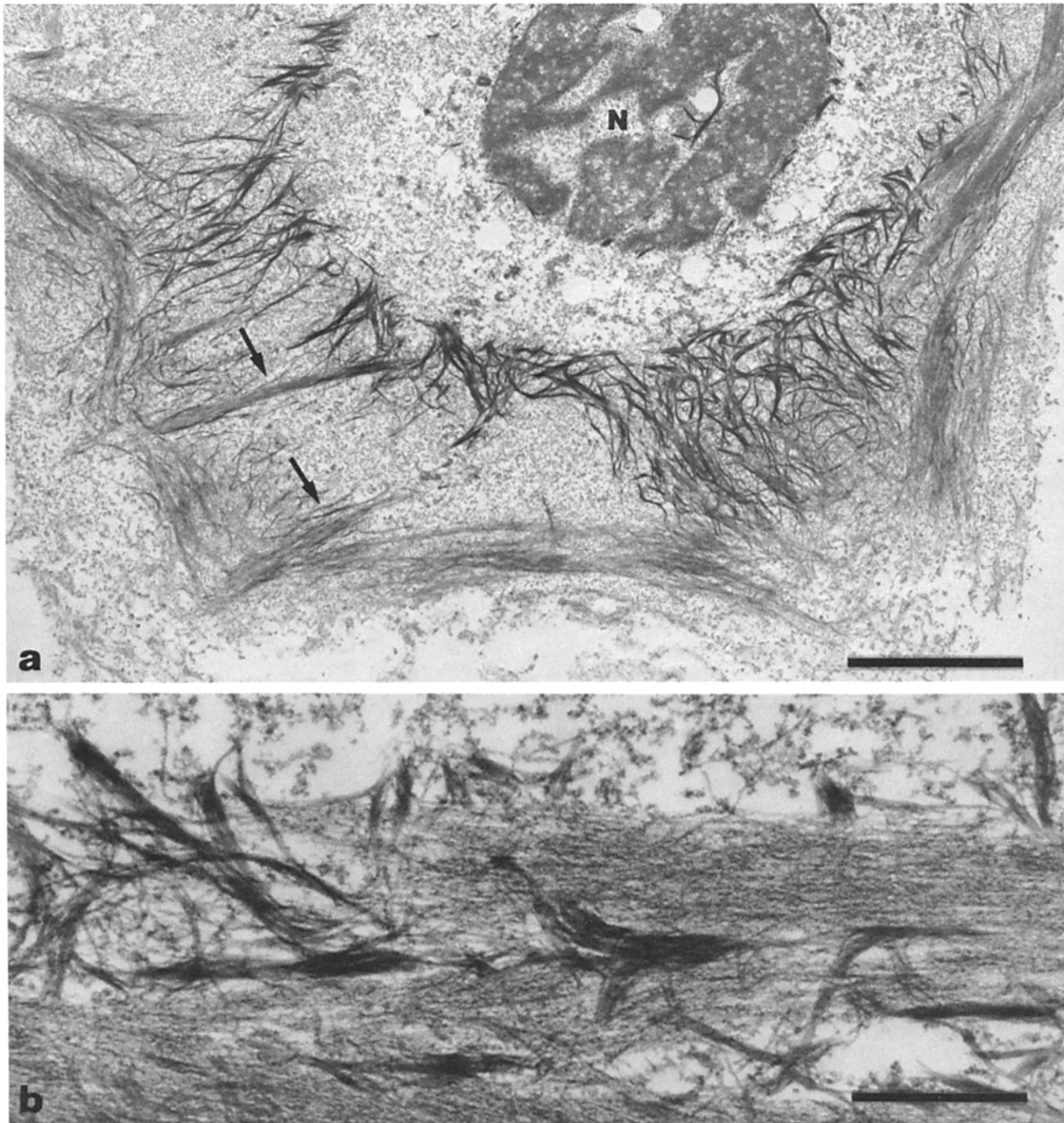


Figure 5. EM of unswitched cells that were lysed and incubated in myosin S-1. (a) Low magnification of a cell containing the nucleus (*N*) and showing electron-dense IFB extending out to the polygonal, peripheral MFB. Note radial, spokelike MFB (*arrows*). (b) Higher magnification of a radial MFB decorated with myosin S-1 showing portions of IFB interwoven into the MFB. Bars: (a) 5 μm ; (b) 0.5 μm .

tions that pass through a ventral-nuclear-dorsal plane suggest that adherens junctions (alternating with desmosomes) predominate along the basal and apical aspects of cell margins at cell-cell interfaces, whereas desmosomes predominate in the center (Fig. 9).

Perturbation of the IF-MF Cytoskeleton with CD

Treatment of PME cells in LCa^{2+} with 0.5 $\mu\text{g/ml}$ CD for various intervals ranging from 30 min to several hours results

in a relatively rapid (within 30–60 min) reorganization of the actin-based cytoskeletal system into numerous patches of fluorescence (Fig. 10 *a*). Double-label fluorescence analysis reveals that the keratin system of IFB is also reorganized into a network with numerous foci, each of which corresponds with an actin patch (Fig. 10 *b*). Ultrastructural analysis confirms the presence of aggregates of amorphous electron-dense material that probably correspond to the actin-containing patches seen in immunofluorescence micrographs around which IF appear to be concentrated (Fig. 10, *c* and *d*). Fre-

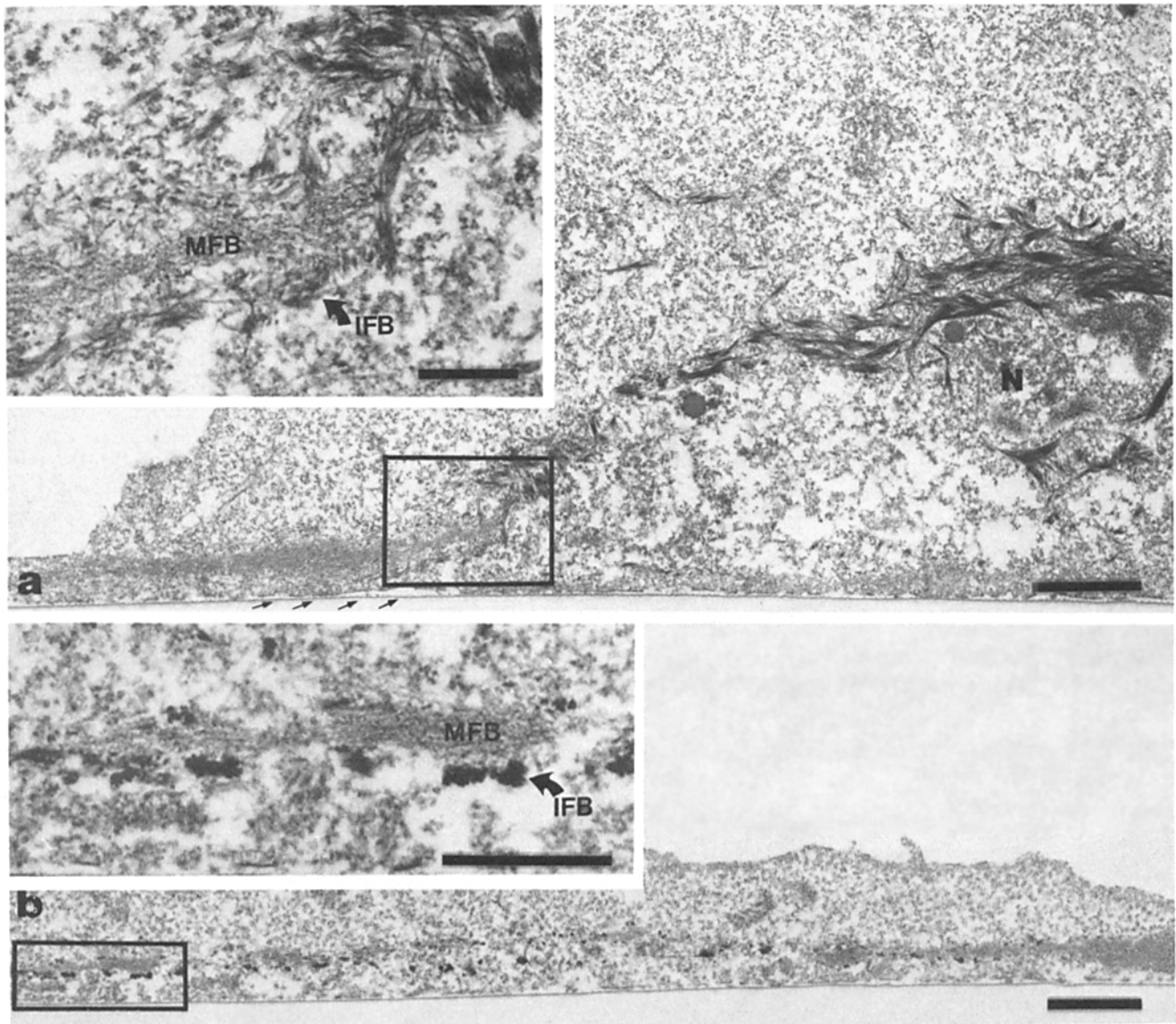


Figure 6. Transverse sections (of unswitched, lysed, S-1-decorated cells) passing through the nucleus (*N*) (*a*) and passing to one side of the *N* (*b*). In *a* note that IFB dive down toward the MFB at the level of the substrate (*small arrows*) where cross-sectional profiles can be seen to be closely associated with MF (*see inset*). In *b* profiles of IFB are in close proximity to MFB at a single level between the dorsal and ventral cell surfaces. Inset shows higher magnification of boxed area. Bars: (*a*) 1 μm ; (*inset*) 0.25 μm ; (*b*) 1 μm ; (*inset*) 0.5 μm .

quently, IFB or IF are seen in transverse section within the aggregates (Fig. 10 *d*, arrows).

Discussion

The data reported here demonstrate the following concerning the PME culture system. (*a*) IF appear to be associated with MF before and during junction formation. IF are related to the circular and radial MFB before and after the Ca^{2+} switch, as well as the MFB that form perpendicular to the region of cell-cell contact during junction formation. (*b*) These latter MFB are associated with actin/vinculin-rich submembranous plaques that exhibit the characteristic ultrastructure of intercellular adherens junctions. (*c*) Adherens junctions, in addition to but independent of desmosomes, are formed at cell-cell interfaces in response to an increase in

external Ca^{2+} concentrations. (*d*) Adherens junction formation and the appearance of short MFB after the Ca^{2+} switch proceeds relatively more quickly than, but is spatially coordinated with, desmosome formation. Adherens junctions were identified by the following criteria: (*a*) anti-vinculin binding, (*b*) association with MFB, and (*c*) characteristic ultrastructure (see 6, 13, 16, 47).

IFB are associated with the circular, concentric MFB, and with radial MFB that intersect the circular MFB, before and after the Ca^{2+} switch. Individual IF appear to splay out from IFB and become interwoven into the MFB. The relationship between IF and the circular MFB is reminiscent of that described by Hull and Staehelin in the terminal web of intestinal epithelial cells. These authors report that IF loop through the network of actin filaments that form a submembranous ring in the subapical adherens region (24). That the

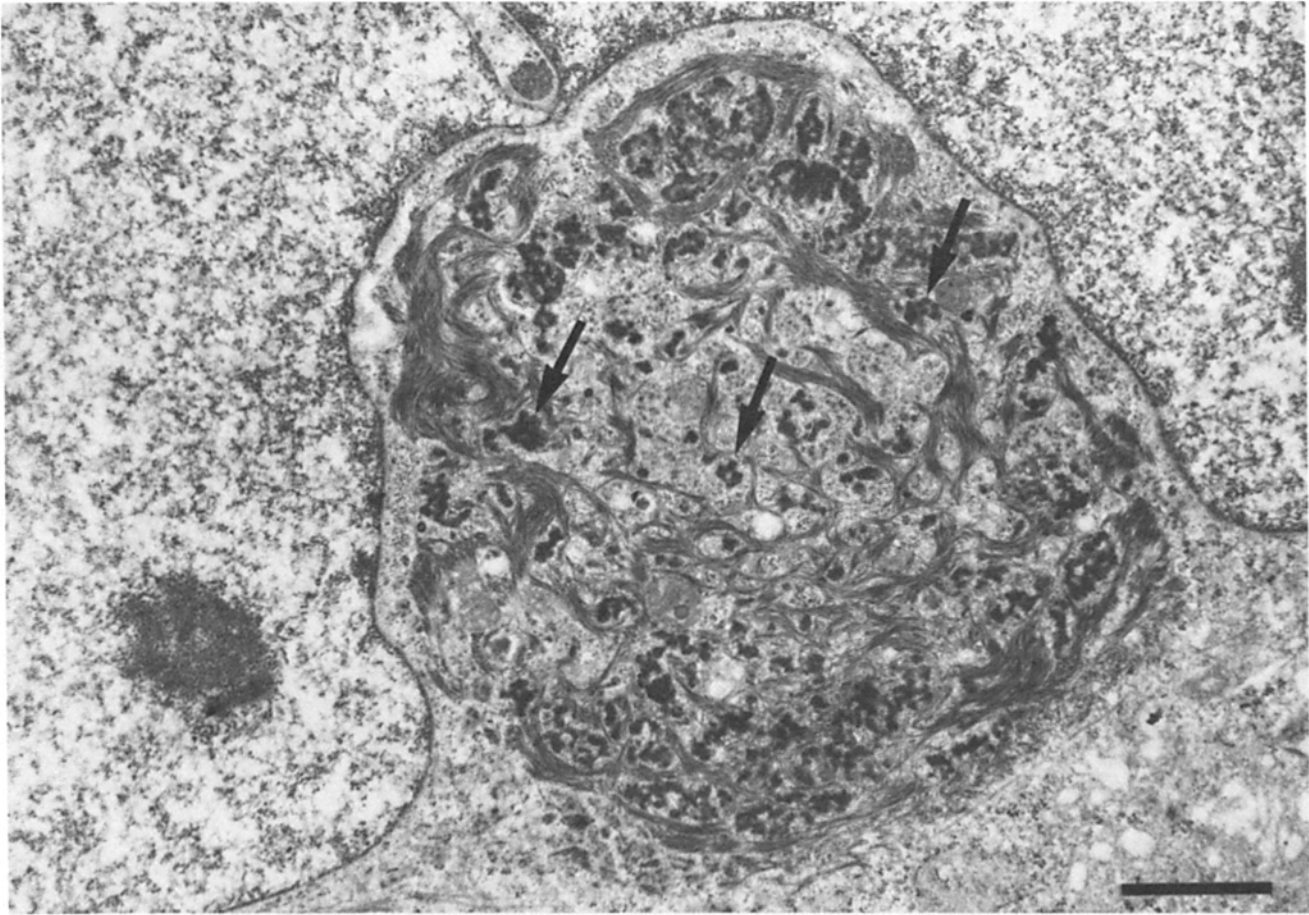


Figure 7. EM of unswitched cell showing an accumulation of desmoplakin-containing bodies (arrows) and IFB near the nucleus. Bar, 1 μ m.

association between IF and MF in PME cells is a fairly intimate one is suggested by CD treatment; under these conditions MF and IF undergo a coordinated, striking rearrangement into actin-containing aggregates that coincide with IF foci. Such a rearrangement of keratin bundles was previously reported by Knapp et al. (31, 32). However, these authors state that a combination of colchicine and cytochalasin was necessary to induce the reorganization. Our results demonstrate that in PME cells CD alone is sufficient (also see references 3 and 52).

The spatial coordination of adherens junctions and desmosomes, i.e., the parallel alignment of MFB and IFB-desmosomal precursor complexes, is very striking. It should be noted here that even though adherens junctions and desmosomes consist of distinct sets of proteins, they often maintain close spatial proximity in mature tissues, such as the terminal bar of polarized epithelia (6, 24) and the intercalated discs of cardiac myocytes (7). Although the molecular basis and functional significance of this organization is not known, the PME system may be useful as a model for understanding how such arrangements are generated. It is tempting to speculate that in PME cells the MFB act as tracks upon which the IFB-desmoplakin complexes translocate or are directed. Our laboratory is currently investigating whether such a model, in which MF play a role in guiding or acting as a motive force for the movement of IFB-desmoplakin complexes, is valid.

At this point it is important to mention the three-dimensional topographical relationship between IFB, MFB, and their associated junctions. That is, adherens junctions (alternating with desmosomes) are located predominantly along the apical and basal aspects of the cell-cell contact zone, and a desmosome-rich region is located in the center. This organization is different from that of polarized epithelia in situ (e.g., intestinal epithelium, references 6 and 24) as well as several cell lines that maintain their polarity in culture (e.g., Madin-Darby canine kidney, Madin-Darby bovine kidney [MDBK], reference 13). As described for intestinal epithelium (above), these cells exhibit an apical belt of a nearly continuous adherens junction associated with a circular MF ring. Desmosomes are located below (ventral to) this ring, as well as along the basolateral cell borders. In the cultured skin keratinocytes, adherens junctions appear to form both above and below desmosomes.

The findings presented here are in agreement with previous reports from Geiger et al. that adherens junctions and desmosomes are distinct entities, each associated with a different cytoskeletal system. In this regard it is interesting to note that an 83-kD protein termed "plakoglobin" that was originally described as a desmosomal component has very recently been localized to plaques of adherens junctions as well (5). Other than the identification of several additional components composing the adherens type of junction (e.g., vinculin, 135 kD, or adherens junction-specific cell adhesion

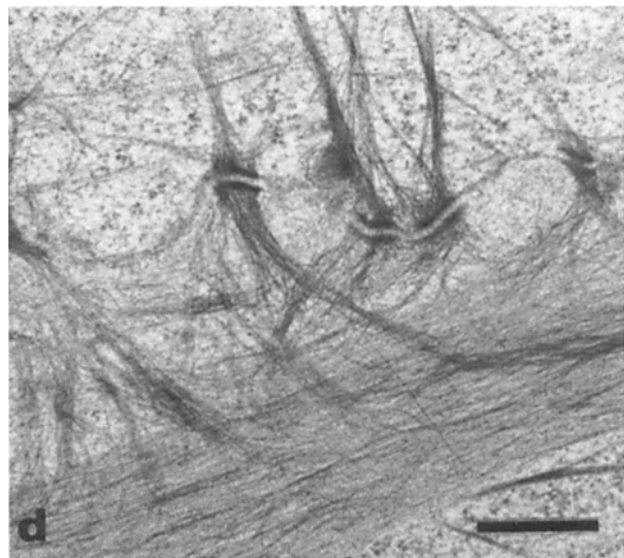
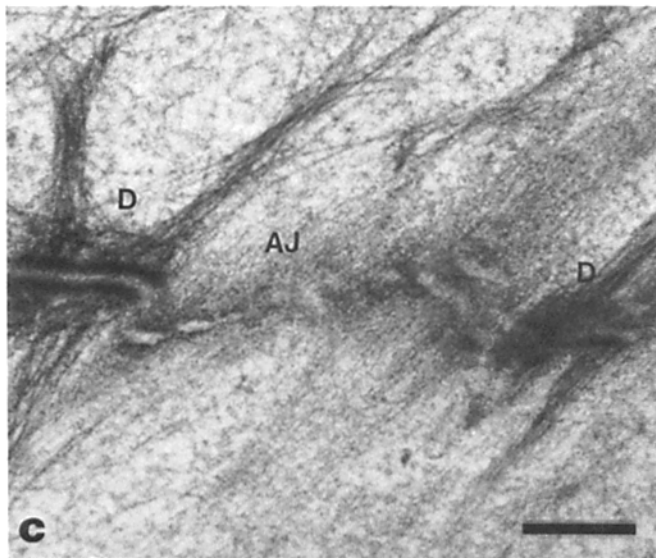
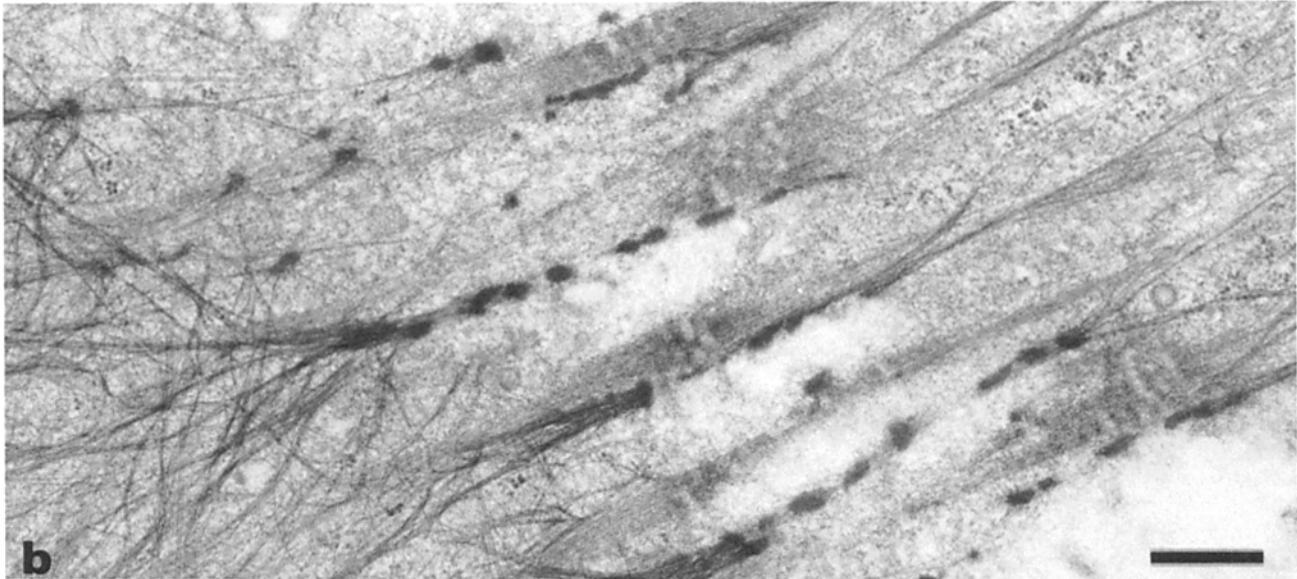
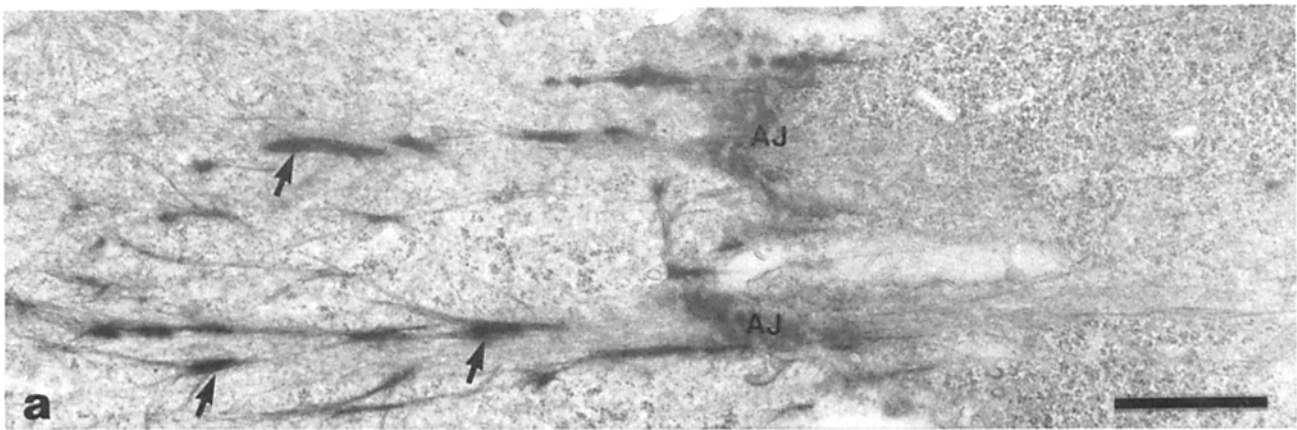


Figure 8. EMs of switched (unlysed) cells in the cell-cell contact region. (a) At 30 min adherens junctions (AJ) but no desmosomes are seen. Electron-dense bodies (arrows; identified by Jones and Goldman, reference 27, as containing desmoplakin) are beginning to align along short MFB. (b) By 2-3 h extensive alignment is seen. (c) After junction formation is complete, desmosomes (D) and adherens junctions (AJ) remain closely associated. (d) IFB can often be seen to extend to desmosomes from peripheral MFB. Bars: (a) 1 μm ; (b) 0.5 μm ; (c) 0.25 μm ; (d) 0.5 μm .

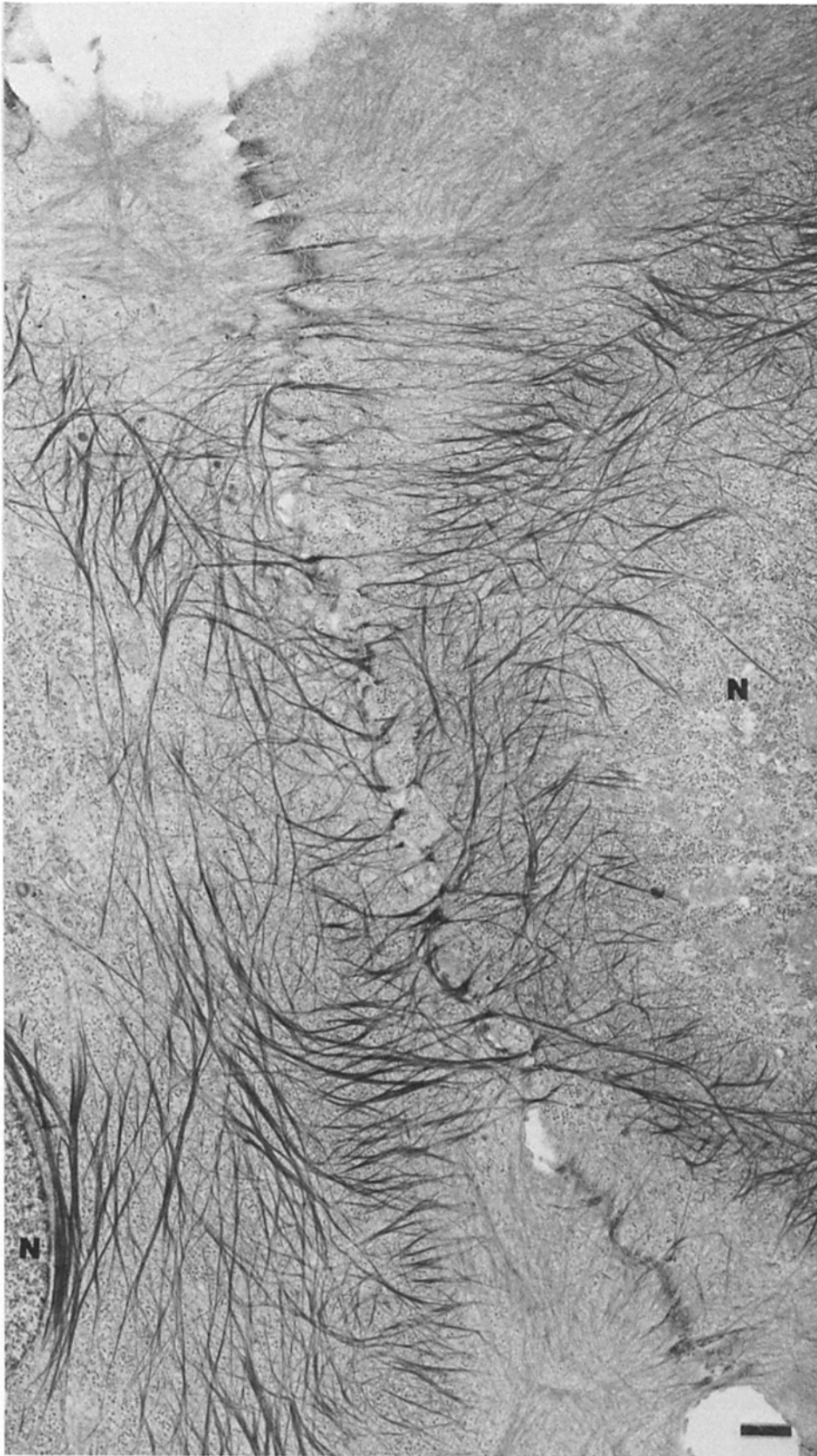
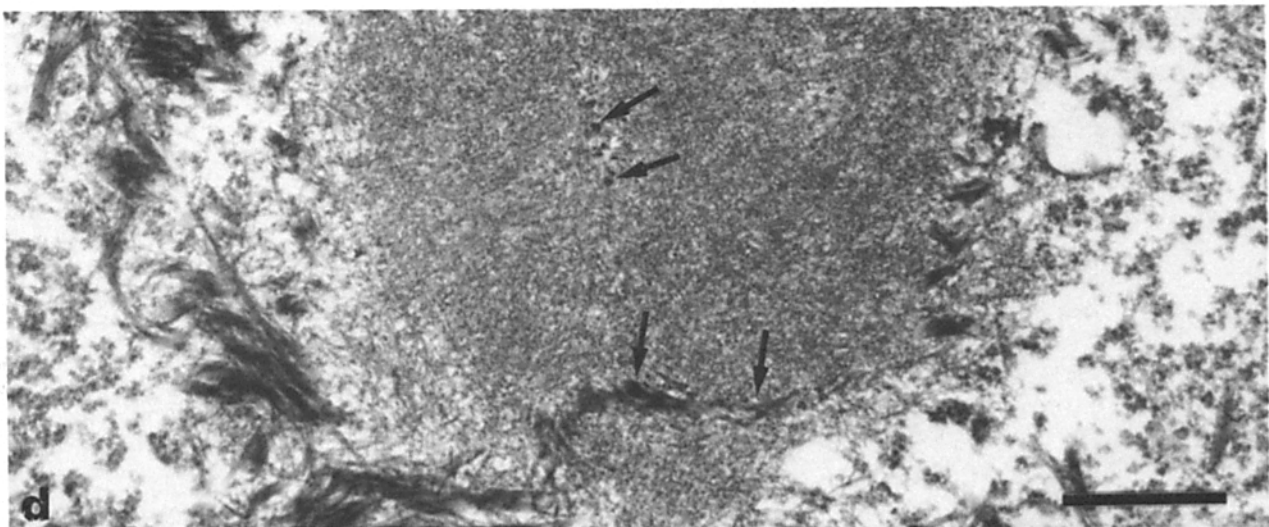
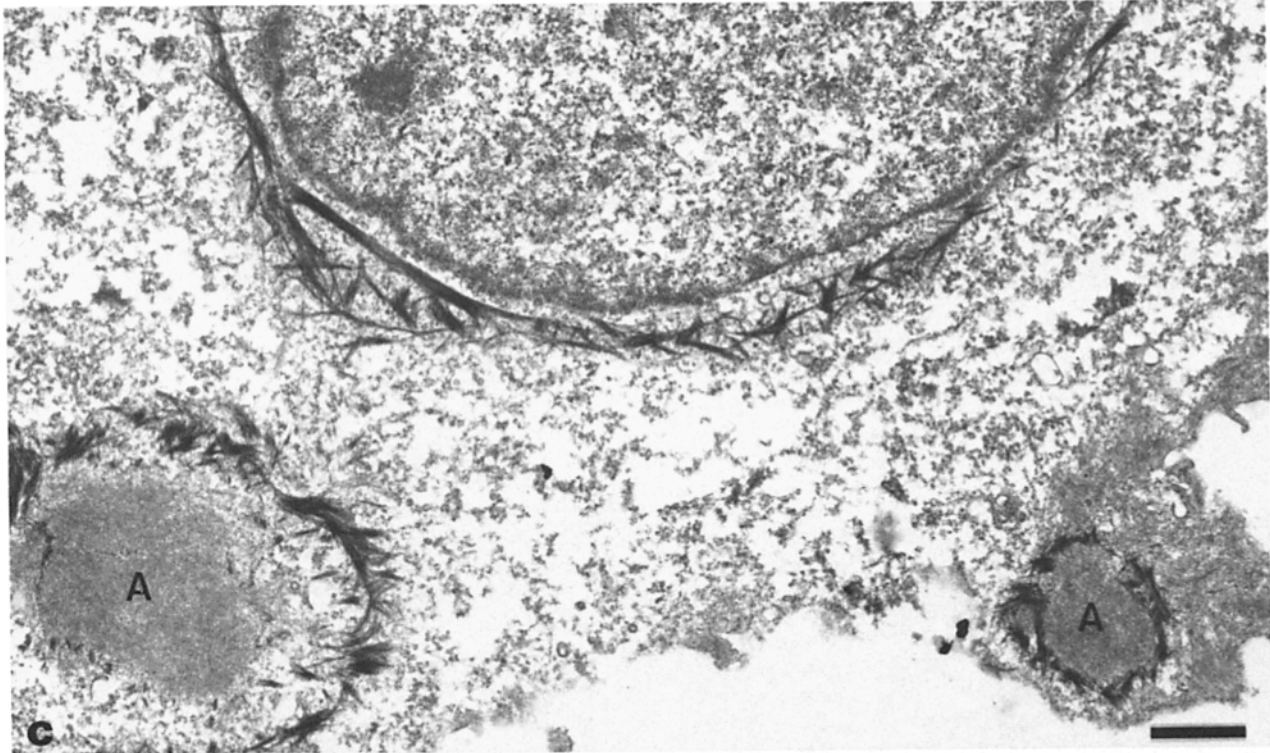
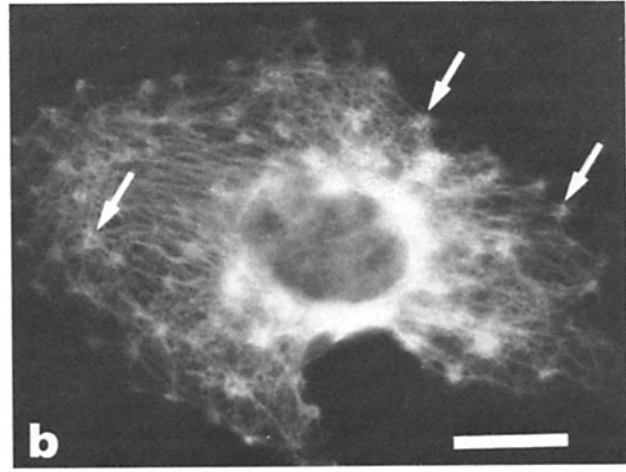
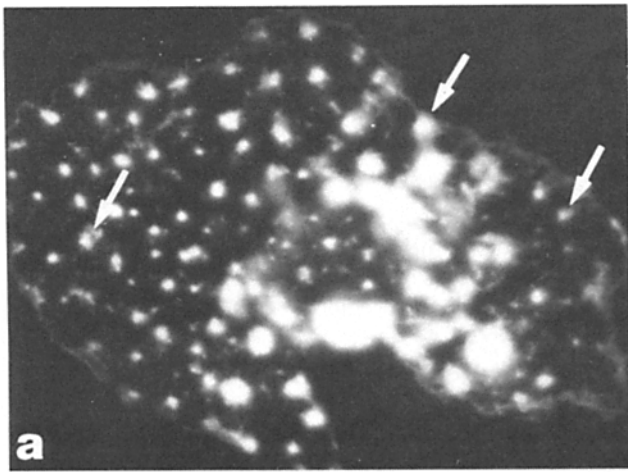


Figure 9. EM of an oblique section passing through ventral-nuclear (*N*)-dorsal plane, showing regions of adherens junctions alternating with desmosomes at the periphery and a desmosome-rich region in the center. Bar, 1 μm .

molecule [A-CAM; found in intercellular contacts], talin [found in cell-substrate contacts], see references 11, 16, 47, 48, 49), little is known about their formation or integration with the MF system. It has, however, been demonstrated for

MDBK cells that, like desmosomes, intercellular adherens junctions are Ca^{2+} dependent, though their fate after EGTA-mediated Ca^{2+} removal appears to be different. Whereas desmosomes are apparently endocytosed (30), adherens



junctions appear to detach as one unit from the membrane (30, 50). These adherens junction remnants apparently can not reattach to the membrane in the renewed presence of Ca^{2+} ; instead they disintegrate in the perinuclear cytoplasm and new junctions are formed only after intercellular contacts are reestablished (50). In addition, it has recently been shown that dissociation and reformation of adherens junctions can be induced in chick lens cells by changing exogenous Ca^{2+} levels (again, using EGTA to remove Ca^{2+}) (49). Furthermore, the formation of adherens junctions is specifically inhibited by monovalent Fab' fragments of antibodies directed against A-CAM. This inhibition is accompanied by abnormal MFB organization. The results suggest a possible role for A-CAM in adherens junction formation (49).

Here we demonstrate that the formation of adherens junctions can also be induced in PME keratinocytes by raising external Ca^{2+} concentrations. Furthermore, we establish a temporal sequence of formation with relationship to desmosome assembly. Within the first 5–30 min after the Ca^{2+} switch, adherens junctions can be detected by immunofluorescence microscopy in many cells. Desmosomes appear to form more slowly: double-label immunofluorescence shows that the redistribution of both desmoplakin and keratin staining lags behind that of actin and vinculin after the Ca^{2+} switch. In this regard, Kartenbeck et al. (30) report that after reestablishment of cell contact in MDBK cells, adherens junctions appear to form much earlier than the desmosomes. The time course of junction formation in PME cells varies somewhat within a cell population; in general, adherens junctions appear between 5–30 min after the switch. Although fully formed desmosomes can be found during this early period, the majority of desmosomes are fully formed between 1–2 (or 3) h after the switch. However, adherens junctions always appear to form before desmosomes at individual cell–cell interfaces.

The assembly of the various elements of the adherens junction is relatively rapid. In data not presented here, the first detectable change after the Ca^{2+} switch is often just an actin/vinculin-rich fluorescent brightening of cell–cell interfaces. Thin MFB seem to appear very shortly after, and subsequently grow in length and width. Geiger (9) has suggested that vinculin-containing contact areas in spreading fibroblasts may act as initiation sites for the formation of actin bundles. This hypothesis is consistent with results reported by Jockush and Isenberg (26), who provided evidence for an interaction between vinculin and F-actin that can induce actin-bundle organization. Recently, Volk and Geiger (49) have extended this hypothesis to include adherens junction formation and have postulated that changes in A-CAM (e.g., aggregation state) may signal the assembly of vinculin, and, ultimately, microfilaments. This hypothesis is consistent with the Ca^{2+} -induced changes in actin and vinculin patterns seen over time in PME cells.

In conclusion, we have shown that in the mouse keratinocyte cell system, MF and IF are closely associated before and after the Ca^{2+} -dependent induction of junction formation.

Furthermore, in addition to desmosomes, adherens junctions also form in response to the increase in external calcium, and the assembly of the two is spatially and temporally coordinated. These results suggest that the PME culture system may provide an opportunity to study the molecular basis for MF–IF interactions and the possible role of MF in the spatial regulation of desmosome formation. The system may also provide an opportunity to study further the mechanism of adherens junction formation, the signals involved in establishing vinculin organization, and the mechanism of contact-induced actin bundle assembly.

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Figure 10. Cells treated with 0.5 $\mu\text{g}/\text{ml}$ CD. Double-label immunofluorescence of an unswitched cell showing the (a) actin (rhodamine-phalloidin) and (b) keratin patterns. Note actin-containing patches coinciding with IF foci (arrows). (c) EM of an unswitched cell showing the nucleus and two aggregates (A), which presumably correspond to the actin patches in a, surrounded by IFB. (d) Higher magnification of one of the aggregates reveals close association with IFB (arrows), which are frequently seen within the aggregates. Bars: (a and b) 10 μm ; (c) 1 μm ; (d) 0.5 μm .

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