Intermediate Filaments and the Initiation of Desmosome Assembly

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ABSTRACT The desmosome junction is an important component in the cohesion of epithelial cells, especially epidermal keratinocytes. To gain insight into the structure and function of desmosomes, their morphogenesis has been studied in a primary mouse epidermal (PME) cell culture system. When these cells are grown in ~0.1 mM Ca^{2+}, they contain no desmosomes. They are induced to form desmosomes when the Ca^{2+} level in the culture medium is raised to ~1.2 mM Ca^{2+}. PME cells in medium containing low levels of Ca^{2+}, and then processed for indirect immunofluorescence using antibodies directed against desmoplakins (desmosomal plaque proteins), display a pattern of discrete fluorescent spots concentrated mainly in the perinuclear region. Double label immunofluorescence using keratin and desmoplakin antibodies reveals that the desmoplakin-containing spots and the cytoplasmic network of tonofibrils (bundles of intermediate filaments [IFB]) are in the same juxtanuclear region. Within 1 h after the switch to higher levels of Ca^{2+}, the spots move toward the cell surface, primarily to areas of cell–cell contact and not to free cell surfaces. This reorganization occurs at the same time that tonofibrils also move toward cell surfaces in contact with neighboring cells. Once the desmoplakin spots have reached the cell surface, they appear to aggregate to form desmosomes. These immunofluorescence observations have been confirmed by immunogold ultrastructural localization. Preliminary biochemical and immunological studies indicate that desmoplakin appears in whole cell protein extracts and in Triton high salt insoluble residues (i.e., cytoskeletal preparations consisting primarily of IFB) prepared from PME cells maintained in medium containing both low and normal Ca^{2+} levels. These findings show that certain desmosome components are preformed in the cytoplasm of PME cells. These components undergo a dramatic reorganization, which parallels the changes in IFB redistribution, upon induction of desmosome formation. The reorganization depends upon both the extracellular Ca^{2+} level and the establishment of cell-to-cell contacts. Furthermore, the data suggests that desmosomes do not act as organizing centers for the elaboration of IFB. Indeed, we postulate that the movement of IFB and preformed desmosomal components to the cell surface is an important initiating event in desmosome morphogenesis.

Desmosomes are intercellular junctions that are presumed to be involved in cellular adhesion (1). They possess a characteristic morphology in which the plasma membranes of two contacting cells associate closely, being separated by a narrow gap containing filamentous material (1). There is a dense plaque structure on the cytoplasmic surface of each half of the desmosome to which bundles of intermediate filaments (IFB)[1] appear to be attached (1). It has been proposed that the desmosome–IFB system allows the transduction of shearing forces from one cell to another (1).

Desmosomes can be disrupted in living cells by treatment

[1] *Abbreviations used in this paper*: IFB, bundles of intermediate filaments; LCa^{2+}, low Ca^{2+} medium (Eagle's minimum essential medium without Ca^{2+} supplemented with 10% chelex-treated serum, 100 U/ml penicillin, and 100 μg/ml streptomycin); NCa^{2+}, normal Ca^{2+} medium (Eagle's minimum essential medium that contains a normal cell culture level of Ca^{2+} supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin); PBSa, phosphate-buffered saline consisting of 6 mM Na^+,K^+ phosphate, 171 mM NaCl, 3 mM KCl, pH 7.4; PME, primary mouse epidermal.

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with proteases or by removal of extracellular Ca^{2+} with chelating agents (2, 3). Re-formation of desmosomes after such treatment has been monitored ultrastructurally (2, 3). Desmosome formation has also been followed *de novo* in a number of developing vertebrate embryos (2, 4) and during wound healing in the epidermis of mice (5). The results of these studies have suggested that the association of IFB with the desmosomal plaque is secondary to the formation of the desmosome itself, i.e., the desmosome acts as some sort of organizing site for the IFB (2–5). Contrary to this, we have reported that desmosomes do not appear to act as organizing centers or polymerization sites for IFB in cultures of mouse keratinocytes in which desmosome formation is controlled by the levels of exogenous Ca^{2+} in the growth medium (6). In this system, mouse keratinocytes can be grown and maintained in low levels of Ca^{2+} (~0.1 mM) for long periods (6). Under these conditions, the keratinocytes do not possess desmosomes. However the epidermal cells can be induced to form desmosomes by an adjustment of the Ca^{2+} concentration to a normal level for growth medium (~1.2 mM) (6, 7). With this system, we have been able to demonstrate that the switch to higher Ca^{2+} initiates a redistribution of IFB from a mainly perinuclear position (6). IFB extend to the cell surface where they appear to associate with desmosomal plaques, which in turn appear to be attached to the plasma membrane.

Our preliminary biochemical observations on the isolated desmosome–IFB complex of cultured mouse keratinocytes suggested that desmosome development involves a reorganization of insoluble proteins (6). In support of this, Hennings and Holbrook (8) have reported that no *de novo* protein synthesis is required for desmosome formation in the mouse keratinocyte culture system. These observations have led us to look for the cellular location of desmosomal proteins in keratinocytes maintained in low levels of Ca^{2+} (i.e., cells that do not possess desmosomes), and also to try to determine whether there is a reorganization of some of these proteins within the cell during desmosome formation. We have done this using antibodies directed against the two so-called desmoplakins (9–11). Desmoplakins 1 and 2 are high molecular weight (250 and 220 kD) protein components associated with the desmosomal plaque, which is located at the inner face of the plasma membrane (9–11). Our results show that desmoplakin is preformed in the cytoplasm of epidermal cells and is reorganized during desmosome formation. Our observations also indicate that desmoplakin 1 appears to be an intermediate filament–associated protein.

MATERIALS AND METHODS

Cell Culture: Primary mouse epidermal (PME) cells were prepared by the trypsin flotation procedure of Yuspa and Harris (12). They were maintained in Eagle's minimum essential medium without Ca^{2+} (Northwestern Media Center, Chicago, IL) supplemented with 10% chelex-treated serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (low Ca^{2+} medium, LCa^{2+}) (7). To induce desmosome formation in PME cells, the LCa^{2+} was removed and replaced with complete Eagle's minimum essential medium (i.e., medium containing a normal cell culture level of Ca^{2+}) with 10% fetal calf serum, 100 U/ml pencillin, and 100 μg/ml streptomycin (normal Ca^{2+} medium, NCa^{2+}) (7).

Antibodies: A rabbit antiserum directed against the desmoplakin 1 (250 kD) component of bovine muzzle desmosomes (9–11, 13) (kindly provided by Drs. James Arnn and Andrew Staehelin) and a rat monoclonal anti-keratin prepared in our own laboratory (14) were used in these studies.

Single Indirect Immunofluorescence: After the cell culture medium was removed, PME cells on glass coverslips were fixed for 2–3 min in −20°C methanol and air dried. Desmoplakin antiserum diluted 1:40 in phosphate-buffered saline (PBSa [6 mM Na^+,K^+ phosphate, 171 mM NaCl, 3 mM KCl, pH 7.4]) or a rat monoclonal antibody directed against mouse keratin (undiluted hybridoma medium) was added to cover the cells. The coverslips were incubated in a moist chamber for 1 h at 37°C, then washed thoroughly in several changes of distilled water. The coverslips were then incubated for a further 30 min at 37°C with fluorescein-conjugated goat anti-rabbit IgG (for the desmoplakin antiserum) or fluorescein-conjugated goat anti-rat IgG (for the monoclonal antibody) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). The coverslips were extensively washed in water and then mounted in Gelvatol (Monsanto Co., St. Louis, MO).

Double Indirect Immunofluorescence: PME cells were processed with the desmoplakin antiserum after fixation in −20°C methanol as described above for single indirect immunofluorescence. However, after an incubation in fluorescein-conjugated goat anti-rabbit IgG, the coverslips were washed extensively in water, and then the cells were re-fixed in 3.7% formaldehyde in PBSa (15). This helped to ensure that the fluorescein-conjugated IgGs did not become redistributed during subsequent incubations, which would contribute to background fluorescence (15). After the coverslips were further rinsed in water, the keratin antibody was overlayed on the cells, and the coverslips were incubated for 1 h at 37°C. After they were washed with distilled water, the coverslips were incubated for an additional 30 min at 37°C with rhodamine-conjugated goat anti-rat IgG (Kirkegaard & Perry Laboratories, Inc.). Coverslips were washed in distilled water and mounted in Gelvatol.

Light Microscopy: A Zeiss Photomicroscope III (Carl Zeiss, Inc., Thornwood, NY) equipped with epifluorescence optics was used for the observation of fixed and stained cells. A Zeiss III RS epiilumination system was equipped with narrow band filter sets for the selective observation of fluorescein and rhodamine. Cells labeled with fluorescein were observed with a xenon 75-W DC lamp, and for those labeled with rhodamine, a 100-W DC mercury arc source was used. Living cells were prepared for observation as reported (6) and maintained at 37°C with a Sage Instruments (Cambridge, MA) Air Curtain incubator. Fluorescence micrographs were taken using Kodak Plus-X film. Phase contrast micrographs were taken using Kodak Panatomic-X film. All films were developed in Diafine (Acufine, Inc., Chicago, IL) two-stage developer.

Electron Microscopy: Cells in petri dishes or pellets of cytoskeletal material (see below) were fixed in 1% glutaraldehyde in PBSa containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ for 30 min, washed 1 h in PBSa, postfixed in 1% OsO_4 in PBSa, rinsed in distilled water, and dehydrated and embedded as previously described (16). Thin sections were made on an LKB Ultratome IV (LKB Instruments, Inc., Gaithersburg, MD) using a diamond knife and were mounted on uncoated copper grids. The grids were stained with uranyl acetate and lead citrate (16). Thin sections were viewed in a JEOL 200 CX electron microscope (JEOL USA, Peabody, MA) at an accelerating voltage of 80 kV. Immunogold localization using the desmoplakin antiserum was done as follows. Cells that grew on petri dishes were fixed for 2 min in −20°C methanol. After they were washed in PBSa, cells were covered with desmoplakin antiserum diluted 1:50 with PBSa and were incubated in a moist chamber for 1 h at 37°C. After four changes of PBSa over a 30-min period, cells were incubated for a further 1 h at 37°C in 5 nm colloidal gold–conjugated goat anti-rabbit IgG (Janssen Pharmaceutical, Beerse, Belgium). After four changes of PBSa over a 30-min period, cells were fixed in 1% glutaraldehyde in PBSa and processed for electron microscopy as above.

Cytoskeleton Preparations: PME cytoskeletons were prepared as described previously (6, 16, 17). In brief, PME cells grown on plastic petri dishes and maintained in LCa^{2+} or switched to NCa^{2+} at various times, were washed twice with PBSa. Cells were then lysed in a high ionic strength, detergent-containing solution (0.6 M KCl, 1.0% Triton X-100, 10 mM $MgCl_2$ in PBSa containing 1 mM phenylmethylsulfonyl fluoride [PMSF, Sigma Chemical Co., St. Louis, MO] and 1 mM *p*-tosyl-L-arginine methyl ester-HCl [TAME, Sigma Chemical Co.]) (6, 16, 17). Lysed cells were removed from petri dishes with a rubber policeman and homogenized by three strokes in a Potter-Elvehjen homogenizer at 4°C. DNAase I (Sigma Chemical Co.) was added (0.5 mg/ml), and the homogenate was incubated for 5 min at 4°C. The residual cell cytoskeletons were pelleted by centrifugation at 2,200 *g* for 5 min in a Beckman TJ-6R centrifuge (4°C) (Beckman Instruments, Inc., Fullerton, CA). The pellet was washed three times in PBSa containing 1 mM PMSF and frozen as a pellet at −80°C for future use. Protein estimations were made by the Bradford procedure (18).

Whole Cell Protein Preparation: One petri dish (100-mm diam) of confluent PME cells was washed briefly with PBSa, and then 1 ml of sample buffer (8 M urea in 0.19 M Tris-HCl, pH 6.8, 1% SDS, 1% β-mercaptoethanol) was added to the cells. The cells were scraped from the dish in the sample buffer. This solution was stirred at room temperature for 30 min and was then stored at −20°C for later use. To estimate the protein concentration of such

preparations, PME cells from comparable confluent 100-mm diam petri dishes were solubilized in 8 M urea in 0.19 M Tris-HCl. Protein was then estimated by the method of Bradford (18).

Western Immunoblotting Procedure: Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS PAGE) using 3–12% gradient acrylamide slab gels with 4.5% acrylamide stacking gels was performed on PME cytoskeletal preparations and total PME cell protein. Both preparations were solubilized in 8 M urea in 0.19 M Tris-HCl, pH 6.8, 1% SDS, 1% β-mercaptoethanol. After this one-dimensional separation, the resulting proteins were transferred to sheets of nitrocellulose paper (19). Immunoblotting was done according to Zackroff et al. (20) using the desmoplakin antibodies.

RESULTS

Light Microscopy

PME cells that grow on glass coverslips in LCa^{2+} do not possess desmosomes even in regions of close cell-to-cell association (6, 7). At various times after such cell cultures are transferred to medium containing NCa^{2+}, desmosome formation proceeds rapidly (6, 7). Cells in LCa^{2+} possess a network of keratin-containing tonofibrils (Fig. 1 *a*), most of which are found in the perinuclear region as determined by indirect immunofluorescence using the monoclonal antibody. Some tonofibrils are also found in the peripheral cytoplasm, but tonofibrils of neighboring cells do not appear to associate at cell–cell contact areas (Fig. 1 *a*). Within 1 h of the Ca^{2+} switch, many of the fluorescent tonofibrils in adjoining cells make contact with the cell surface (Fig. 1 *b*), specifically in areas of cell–cell contact (Fig. 1 *b*). 2 h after the Ca^{2+} switch, large numbers of keratin-containing tonofibrils are associated

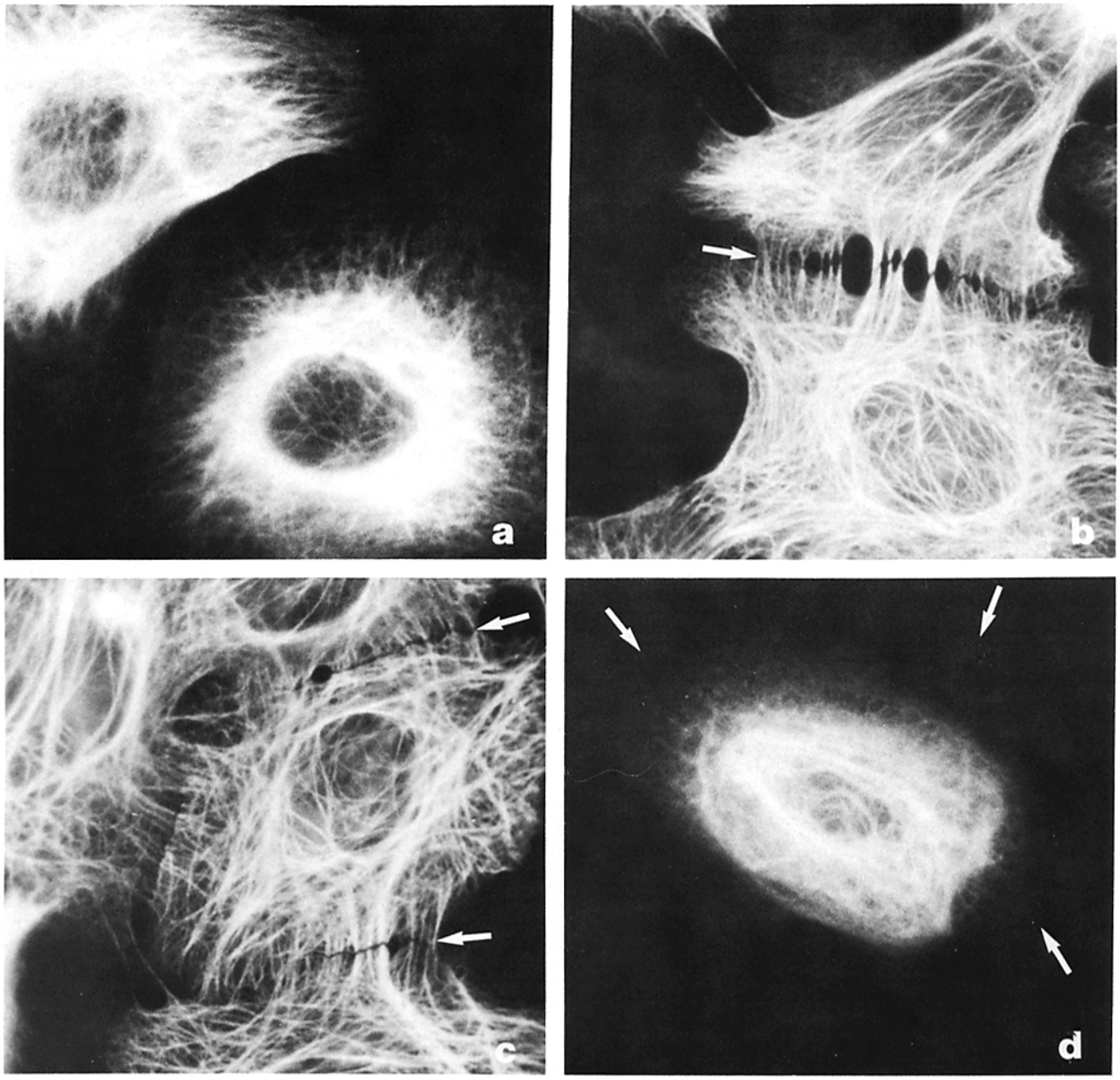

FIGURE 1 Keratinocytes were prepared for indirect immunofluorescence using the monoclonal antikeratin before (a), 1 h (*b*), and 2 h (c and *d*) after the switch to NCa^{2+}. In LCa^{2+}, the keratin-containing tonofibrils appear to be concentrated in the perinuclear zone (*a*). After treatment with NCa^{2+} (*b*), the tonofibrils move from this region and appear to establish contact with the cell surface (*b*, arrow) Tonofibrils in contacting cells are separated by a thin nonfluorescent band (*b* and c, arrows). In single keratinocytes (i.e., a cell possessing no contacts with neighboring cells), even 2 h after the switch from LCa^{2+} to NCa^{2+} (*d*), no obvious reorganization of the tonofibrils is seen. The edge of the cell is labeled with arrows. × 2,500.

with the contiguous borders of neighboring cells where they are separated by a thin nonfluorescent band (Fig. 1 *c*). In single cells that are not in contact with other cells, even up to 2 h after the Ca^{2+} switch, no obvious redistribution of the tonofibrils from the perinuclear region can be detected (Fig. 1 *d*).

Desmosome formation was monitored in PME cells by indirect immunofluorescence using the desmoplakin antiserum. In PME cells maintained in LCa^{2+}, the desmoplakin antiserum stains spots that are located near the substrate-attached surface of the cell (Fig. 2, *a* and *b*). These spots are distributed mainly over the central region of the cell and are rarely found in the peripheral cytoplasm (Fig. 2, *a* and *b*). Within 1 h of the Ca^{2+} switch, the desmoplakin-staining spots become more concentrated near cell-to-cell contact areas (Fig. 2 *c*). Strands or rows of the desmoplakin-staining spots appear in regions leading to cell–cell contact areas (Fig. 2, *c* and *d*). These spots appear to aggregate at the cell surface (Fig 2 *c*). 2 h after the Ca^{2+} switch, nearly all of the detectable fluorescence observed using the desmoplakin antibody appears in areas of cell-to-cell contact marking the position of desmosomes (Fig. 3, *a* and *b*). No fluorescence is observed at free edges of cells (Fig. 3, *a* and *b*), i.e., the reorganization of the desmoplakin-staining spots detected appears polarized and occurs only in the transcytoplasmic region between the nuclear and cell surfaces adjacent to cell–cell contacts (Fig. 3, *a* and *b*). As in the case of the tonofibrils in single PME cells without cell contacts even 2 h after the Ca^{2+} switch, there is no obvious reorganization of the desmoplakin-staining spots from a perinuclear position to the cell surface (Fig. 3, *c* and *d*).

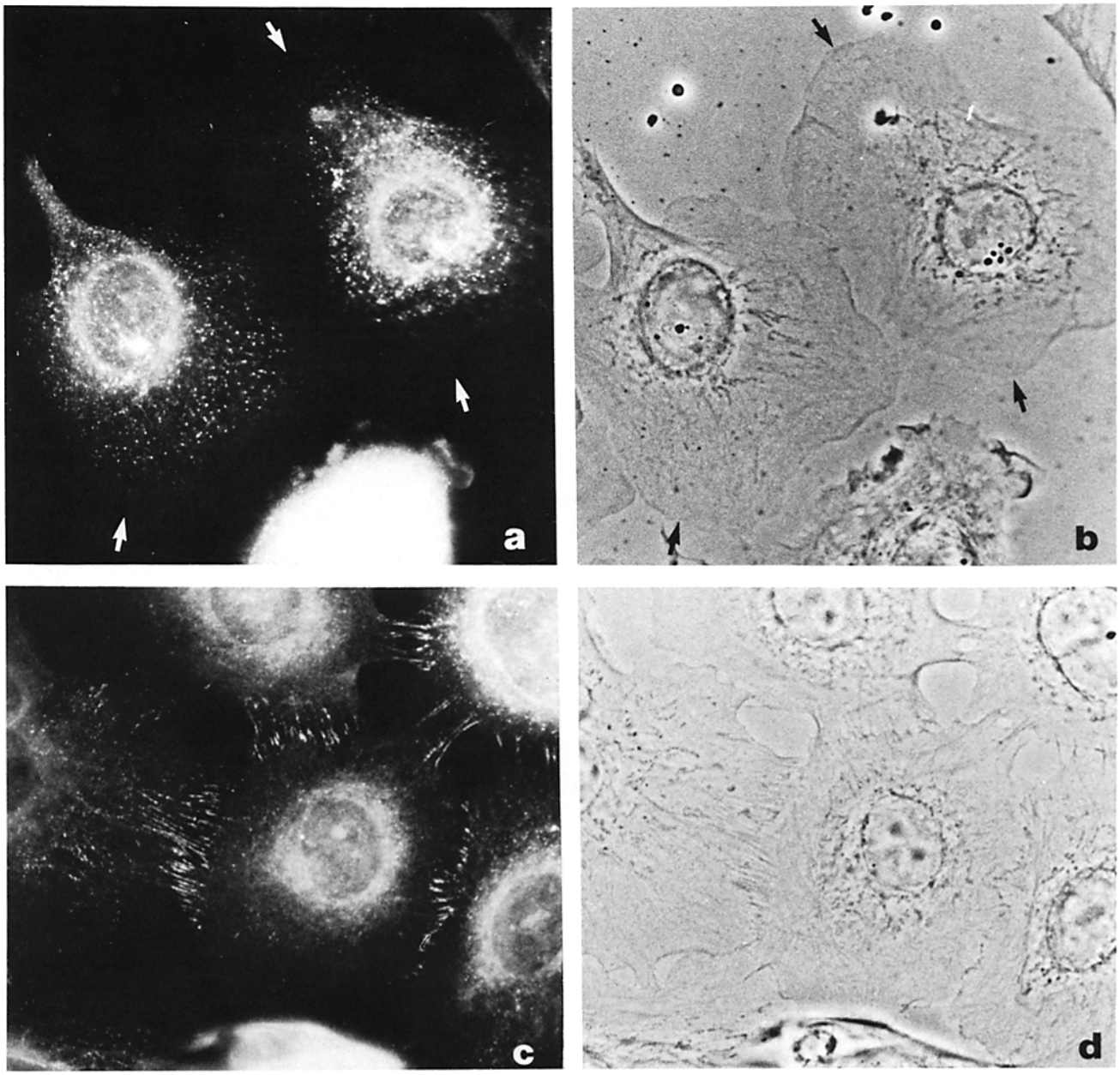

FIGURE 2 Keratinocytes were prepared for indirect immunofluorescence using the desmoplakin antiserum before (*a* and *b*) or 1 h (*c* and *d*) after the switch to NCa^{2+}. In LCa^{2+}, the desmoplakin antiserum stains discrete spots that are located near the substrate-attached surface of the cell mainly in the perinuclear region and that do not associate with the cell surface (*a* and *b*, arrows). Within 1 h after the Ca^{+} switch, the desmoplakin-staining spots appear to become more concentrated near cell–cell contact areas (*c* and *d*). × 1,750.

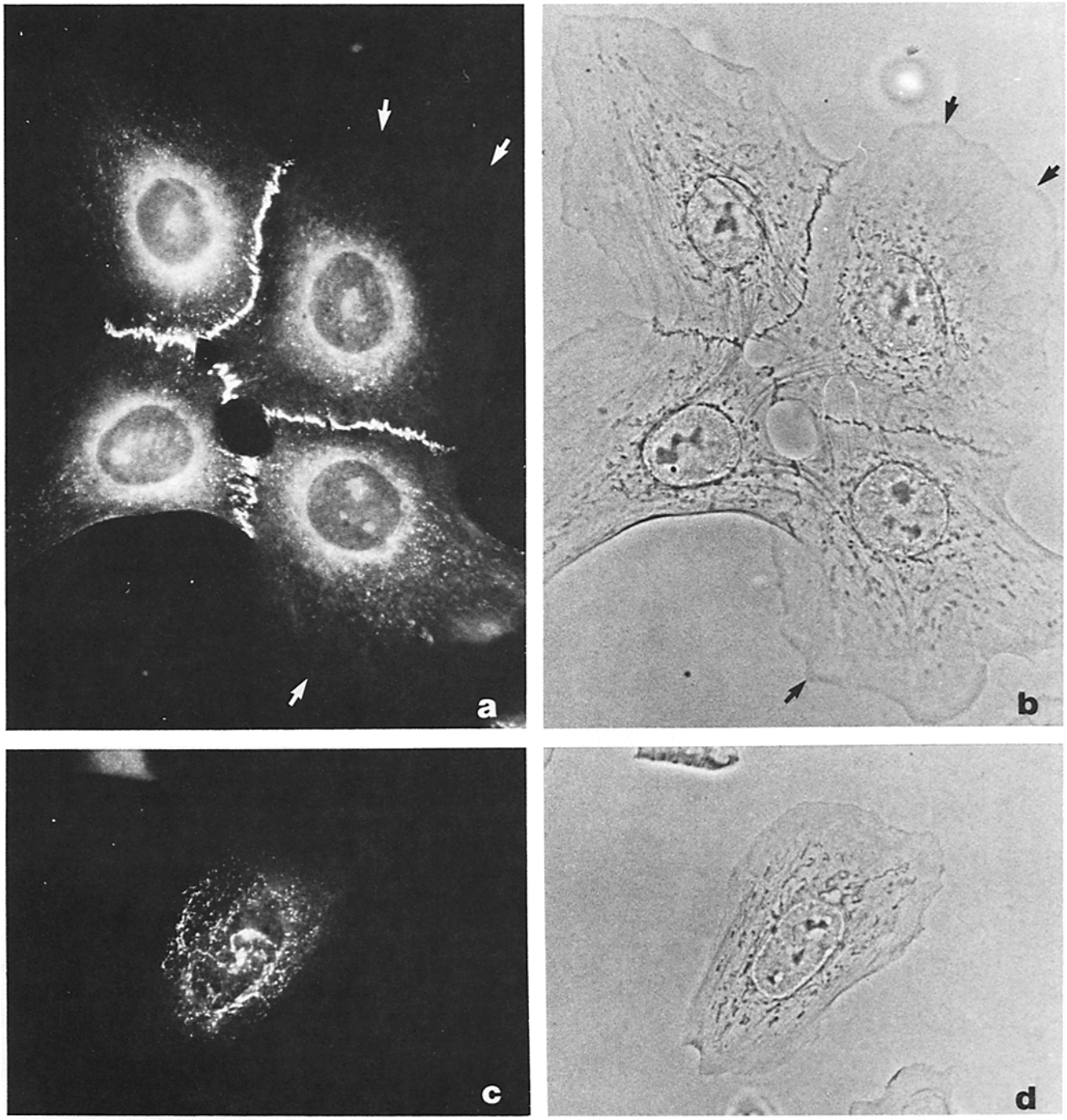

FIGURE 3 Keratinocytes were prepared for indirect immunofluorescence using the desmoplakin antiserum 2 h after the switch to NCa^{2+}. The desmoplakin antiserum primarily stains the borders of contiguous cells (*a*), marking the position of desmosome visualized as phase dense plaques in phase contrast microscopy (*b*), and does not stain free edges of the cells (arrows). In single keratinocytes (*c* and *d*) (i.e., a cell possessing no contacts with other cells) even 2 h after the switch to NCa^{2+}, the desmoplakin-staining spots remain primarily in their perinuclear position and do not undergo any obvious reorganization. × 1,750.

To define more precisely the association between tonofibrils and desmoplakin, PME cells maintained in LCa^{2+} and those switched for 2 h to NCa^{2+} were processed for double indirect immunofluorescence using the desmoplakin antiserum and the monoclonal anti-keratin. In PME cells maintained in LCa^{2+}, the fluorescent spots detected with the desmoplakin antiserum, in some instances, appear to be aligned along the fluorescent tonofibrils (Fig. 4, *a* and *b*). 2 h after the Ca^{2+} switch, many of the desmoplakin spots have redistributed to areas of cell–cell contact where tonofibrils of neighboring cells closely associate (Fig. 4, *c* and *d*). The band of fluorescence observed using the desmoplakin antiserum at areas of cell–cell contact, marking the position of desmosomes, is coincident with the nonfluorescent region between associating tonofibrils along the borders of contiguous cells (Fig. 4, *c* and *d*).

We determined whether PME cells underwent any dramatic change in shape after the Ca^{2+} switch. This was to rule out the possibility that a change in cell shape could account for

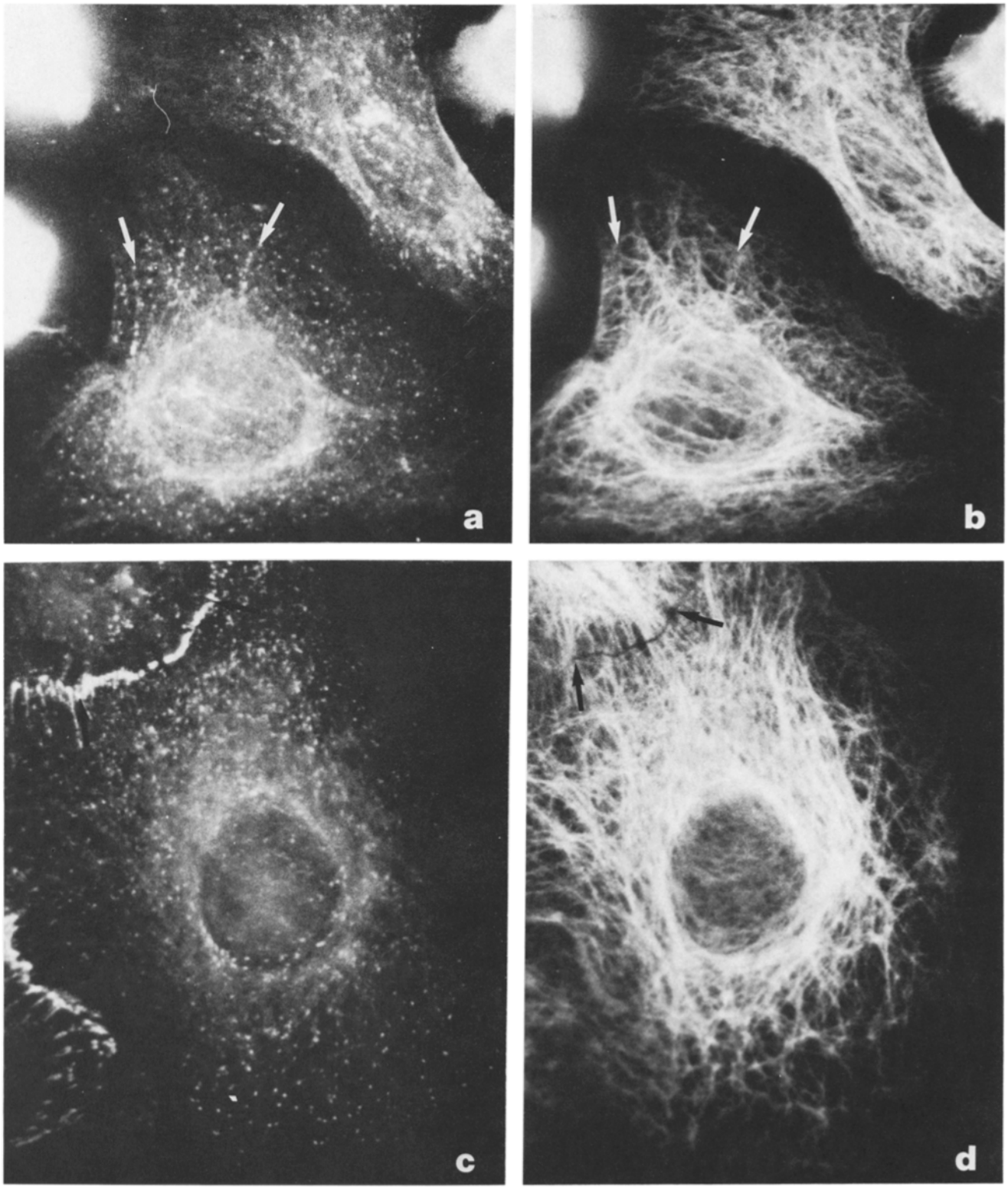

FIGURE 4 Keratinocytes were prepared for double indirect immunofluorescence using the desmoplakin antiserum (*a* and *c*) and the monoclonal anti-keratin (*b* and *d*) both before (*a* and *b*) and at 2 h (*c* and *d*) after the induction of desmosome formation in NCa^{2+}. The desmoplakin-staining spots observed in *a* appear to co-localize with the tonofibrils (*b*, arrows). The nonfluorescent area detected between associating tonofibrils of contiguous cells (*d*, between arrows) is stained by the desmoplakin antiserum (*c*, between arrows). Note that the reorganization of desmoplakin and tonofibrils during desmosome formation occurs specifically to areas of cell–cell contact and not in areas adjacent to the cell borders that are free of contacts with neighboring cells (*c* and *d*). × 2,500.

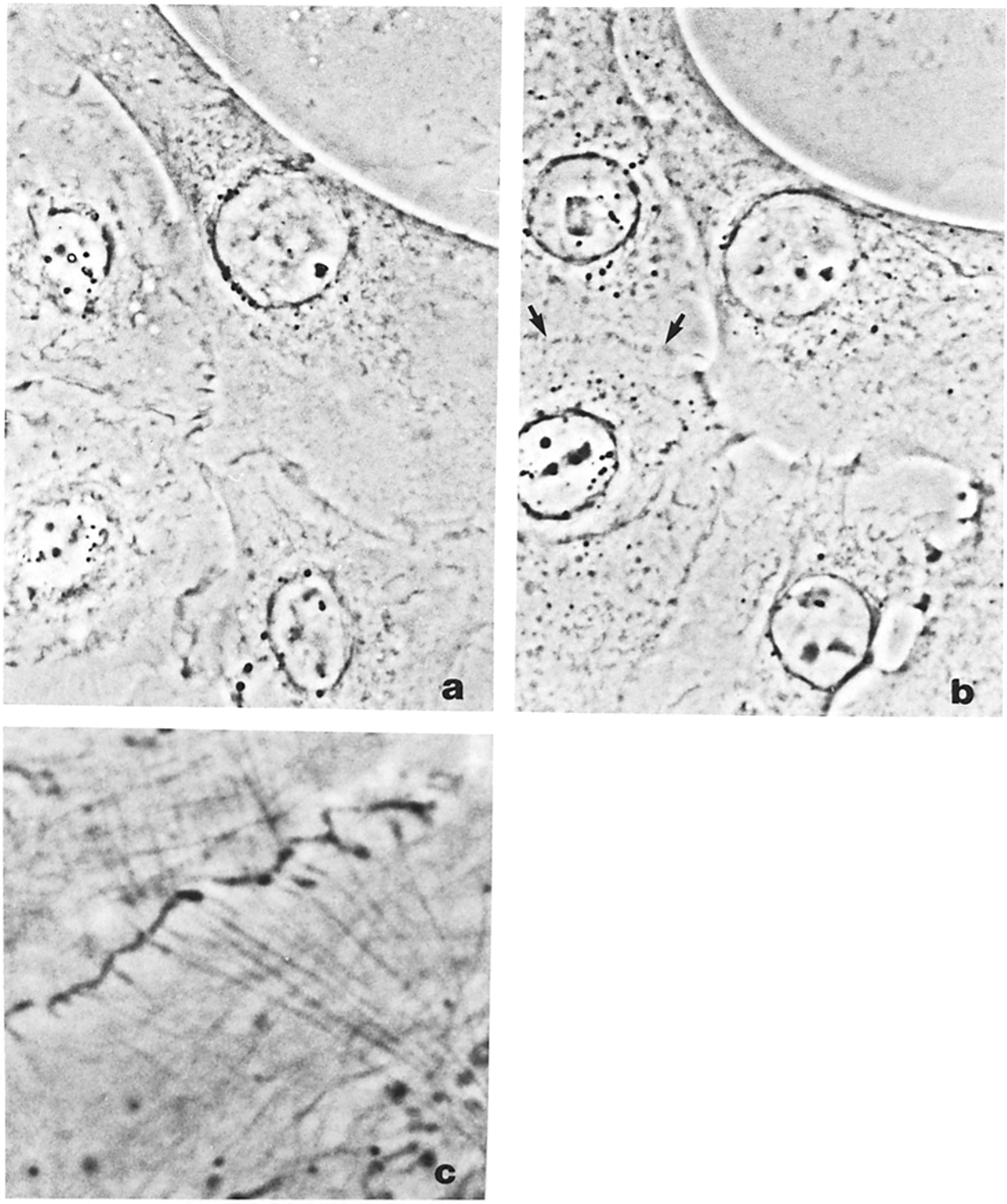

FIGURE 5 Live keratinocytes were monitored by phase contrast microscopy in LCa^{2+} (*a*) and 5 h after the switch to NCa^{2+} (*b* and *c*). An area of cell contact (arrow in a) is shown at high magnification in c. Phase dense plaques with associated phase dense tonofibrils are seen along an area of cell–cell contact (*c*). (*a* and *b*) × 1,800; (*c*) × 3,000.

the reorganization of both the keratin-containing tonofibrils and the desmoplakin-staining spots that we have described above. Live PME cells grown on coverslips were monitored by phase contrast microscopy in LCa^{2+} and during a 5-h period after the Ca^{2+} switch. The same cells maintained in LCa^{2+} are shown in Fig. 5*a*, and 5 h after the Ca^{2+} switch in Fig. 5*b*. 5 h after the Ca^{2+} switch, phase dense plaques representing the position of desmosomes (Fig. 5*c*; see also references 6 and 15) are visible along a cell–cell contact area. Although the cells have moved slightly during the 5-h incubation period, no dramatic flattening or rounding of the cells has occurred (compare Fig. 5, *a* and *b*).

Electron Microscopy

Ultrastructural techniques were used to investigate the pos-

sible structural relationship between the desmoplakin-enriched spots and the keratin-containing tonofibrils detected by indirect immunofluorescence. Thin sections of PME cells grown in LCa^{2+} were prepared and studied by electron microscopy. These reveal that the tonofibrils observed by fluorescence microscopy can be most readily accounted for by IFB (Fig. 6 *a*). Thin sections taken toward the substrate-attached cell surface of PME cells before the Ca^{2+} switch reveal that many IFB are associated with electron dense amorphous bodies (Fig. 6, *a* and *b*), which probably account for the fluorescent spots detected in PME cells in LCa^{2+} using the desmoplakin antiserum (see above). In PME cells switched to NCa^{2+}, these bodies appear to move closer to areas of cell–cell contact (Fig. 6 *c*). Strings of these bodies in close association with IFB appear to be in the process of forming desmosomes and can be seen along the borders of contiguous cells (Fig. 6 *d*).

To determine whether or not these amorphous, electron dense bodies contain desmoplakin, PME cells were processed in LCa^{2+} and at 2 h after the switch to NCa^{2+} using ultrastruc-

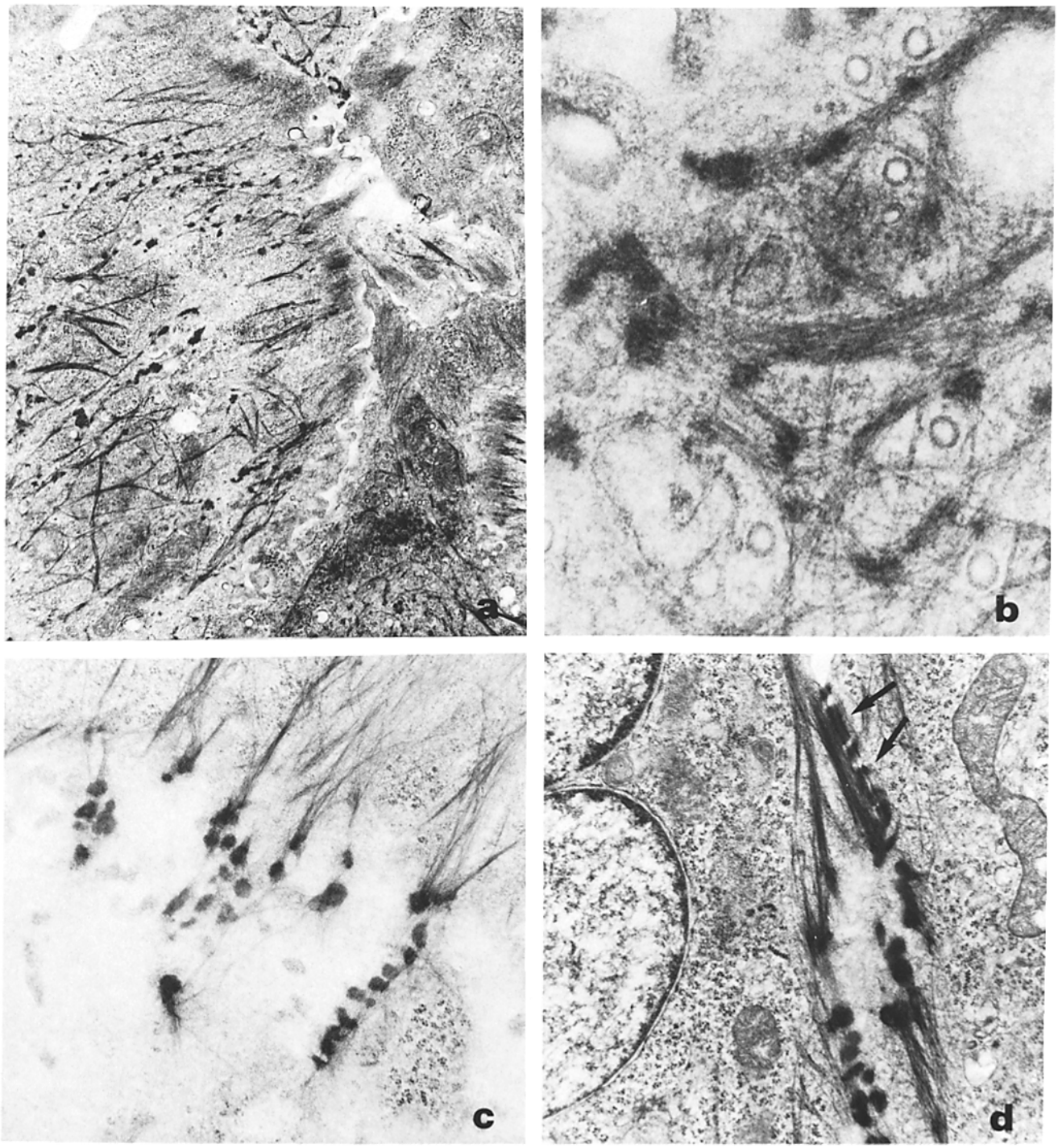

FIGURE 6 Thin sections taken near the substrate-attached surface of keratinocytes before the Ca^{2+} switch reveal that IFB are associated with electron dense amorphous bodies (*a* and *b*). In keratinocytes 1 h after the induction of desmosome formation, these bodies appear to migrate to areas of cell–cell contact (*c*). Strings of these bodies in close association with IFB, as well as desmosomes, are visible along the borders of contiguous cells 2 h after the Ca^{2+} switch (*d*). (*a*) × 11,000; (*b*) × 93,000; (*c*) × 21,500; (*d*) × 19,600.

tural immunogold localization of desmoplakin antibodies. In LCa^{2+}, gold particles can be found primarily at the periphery of the amorphous bodies associated with IFB (Fig. 7 *a*). After 2 h in NCa^{2+}, the gold particles remain in close association with these bodies, which have moved closer to the cell surface along with their associated IFB (Fig. 7 *b*). At this time, gold particles are associated with forming desmosomes primarily in the region of the dense desmosomal plaque to which IFB appear to be attached (Fig. 7 *c*).

Biochemical Analyses

Cytoskeletal preparations of PME cells grown in LCa^{2+}, or after their transfer to NCa^{2+} for 2, 4, and 24 h have been analyzed by electron microscopy and SDS PAGE. Such preparations isolated from PME cells maintained in LCa^{2+} consist primarily of IFB associated with amorphous electron dense bodies (Fig. 8 *a*). However, no obvious desmosomes are present. 24 h after the Ca^{2+} switch, the cytoskeletal preparations isolated from PME cells also consist mainly of IFB; however, these preparations contain very few amorphous dense bodies, but there are numerous desmosomes (Fig. 8 *b*).

Analysis of these cytoskeletal preparations by SDS PAGE reveal that they contain major polypeptides of 60, 53, and 48 kD (Fig. 9). These polypeptides have been shown to be

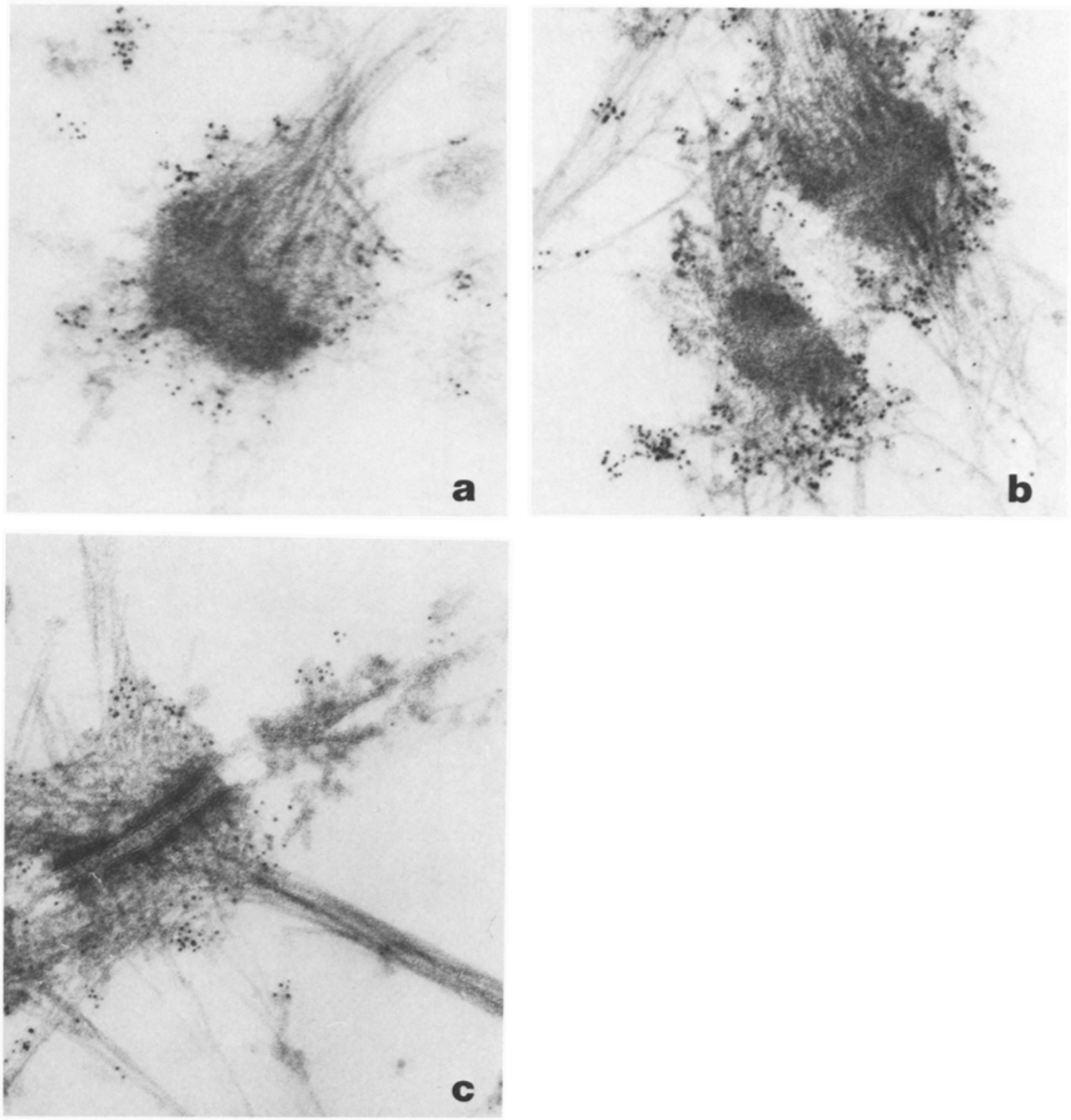

FIGURE 7 Keratinocytes both before and after the induction of desmosome formation were processed for immunogold ultrastructural localization using the desmoplakin antiserum. In LCa^{2+}, gold particles are seen primarily around the periphery of the electron dense amorphous bodies associated with IFB (*a*). 2 h after the induction of desmosome formation, gold particles remain in close association with these bodies that have moved closer to the cell surface with their associated IFB (*b*). In fully formed desmosomes 2 h after the Ca^{2+} switch, gold particles are seen in areas where IFB associate with the desmosomal plaque (*c*). × 96,500.

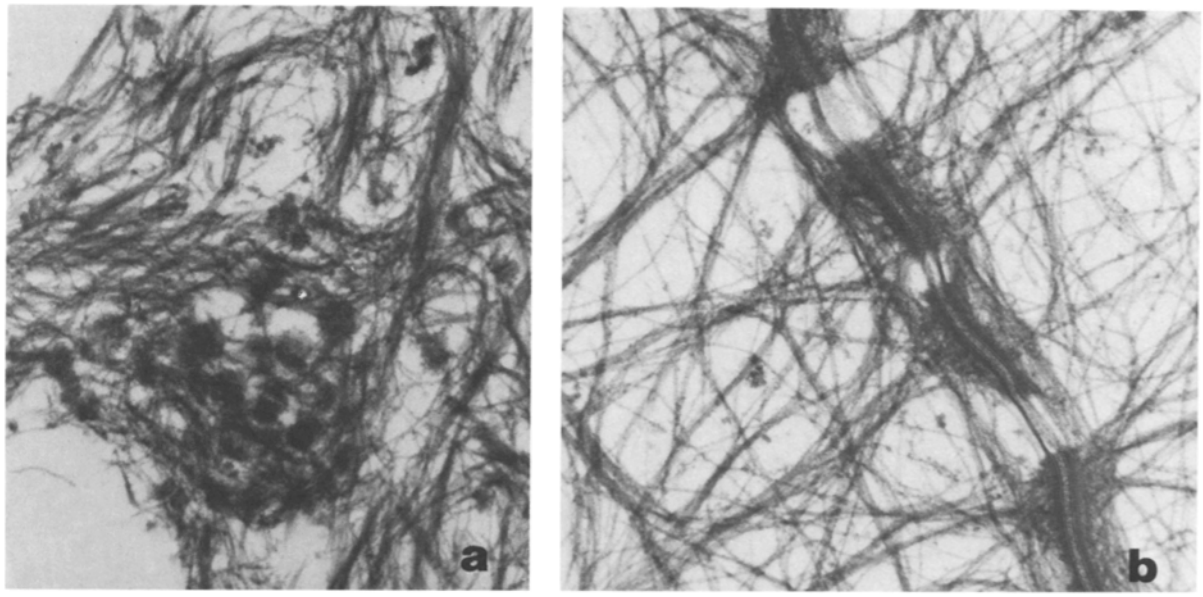

FIGURE 8 Cytoskeleton preparations isolated from keratinocytes maintained in LCa^{2+} consist primarily of IFB that are associated with dense amorphous bodies (*a*). 24 h after the induction of desmosome formation, the cytoskeletal preparation of keratinocytes consists mainly of IFB and desmosomes with very few amorphous bodies being evident (*b*). × 72,400.

keratins (15). However, the polypeptide profiles of the cytoskeletal preparations isolated at different times before and after the Ca^{2+} switch differ in their minor protein components. All of the cytoskeletal preparations possess a polypeptide migrating at 250 kD, but there appears to be relatively increasing amounts of this protein as a function of time after the switch to NCa^{2+} (Fig. 9). Furthermore, the polypeptide migrating at 220 kD is not detectable by SDS PAGE in cytoskeletons isolated from cells maintained in LCa^{2+}, but appears in preparations isolated from PME cells that had been switched to NCa^{2+} for 2 h or more (Fig. 9). Both the 250 and 220 kD polypeptides appear to be maximally enriched in cytoskeletons after 24 h in NCa^{2+} (Fig. 9).

We wondered whether the differences in polypeptide composition in the cytoskeletal preparations during desmosome formation were due to changes in the desmoplakins. We thus attempted to further characterize the high molecular weight proteins by the Western blotting technique (Fig. 10). Whole cell protein extracts and cytoskeletal preparations were subjected to SDS PAGE, electrophoretically transferred to nitrocellulose, and reacted with the desmoplakin 1 antiserum. In the whole cell protein preparations of both PME cells maintained in LCa^{2+} and those switched for 24 h to NCa^{2+}, the desmoplakin antiserum reacts with two proteins of 250 and 220 kD (Fig. 10, lanes *C* and *D*). Therefore, desmoplakin 1 antiserum appears to react with both desmoplakins 1 and 2. This is not surprising, as Mueller and Franke (10) have shown that there is considerable homology between the desmoplakins as determined by two-dimensional peptide maps, and these same authors have already reported that a polyclonal antiserum to an individual desmoplakin reacts with both desmoplakins 1 and 2. In the cytoskeletal preparation isolated from PME cells grown in LCa^{2+}, the desmoplakin antiserum reacts only with a 250-kD polypeptide (i.e., desmoplakin 1) (Fig. 10, lane *G*). In the cytoskeletal preparation isolated from cells in whch desmosome formation had been induced by the Ca^{2+} switch, the desmoplakin antiserum reacts with both a 250 and 220-kD polypeptide (i.e., both desmoplakins 1 and 2) (Fig. 10, lane *H*).

DISCUSSION

The mouse epidermal cell culture system of Hennings et al. (6) has been valuable in the elucidation of the developmental sequence of desmosome formation. We have already reported that desmosome formation can be monitored in live mouse keratinocytes by phase contrast microscopy (6). Desmosome formation has also been followed in this system by electron microscopy (6, 8). In the present study, we have used antibodies made against desmoplakin obtained from bovine muzzle epidermis to study stages in the formation of desmosomes both morphologically and biochemically.

Both desmoplakin and IFB undergo dramatic reorganization when PME cells are switched to NCa^{2+}. IFB are redistributed from a mainly perinuclear position and ultimately establish associations with the cell surface (6). Concomitantly, the desmoplakin-containing spots or bodies move toward cell-to-cell contact sites. Indeed, the reorganization of IFB and desmoplakin appears to be coordinated and occurs specifically in areas adjacent to contact regions and not in areas that are free of such contacts. Furthermore, there appears to be little, if any, reorganization of IFB and desmoplakin in cells grown in NCa^{2+} that lack cell–cell contacts. Thus, our results suggest that the redistribution of desmoplakin and IFB depends not only upon the extracellular level of Ca^{2+} but upon the existence or establishment of cell-to-cell contacts.

Using the desmoplakin antiserum, we have shown that desmoplakin is present in whole cell extracts of PME cells maintained in LCa^{2+} (i.e., in cells that lack desmosomes). This result, taken together with our morphological observations, suggests that desmoplakin is preformed in the cytoplasm and is a component of discrete electron dense bodies associated with IFB before desmosome formation. When keratinocytes receive the correct clues or signals from their environ-

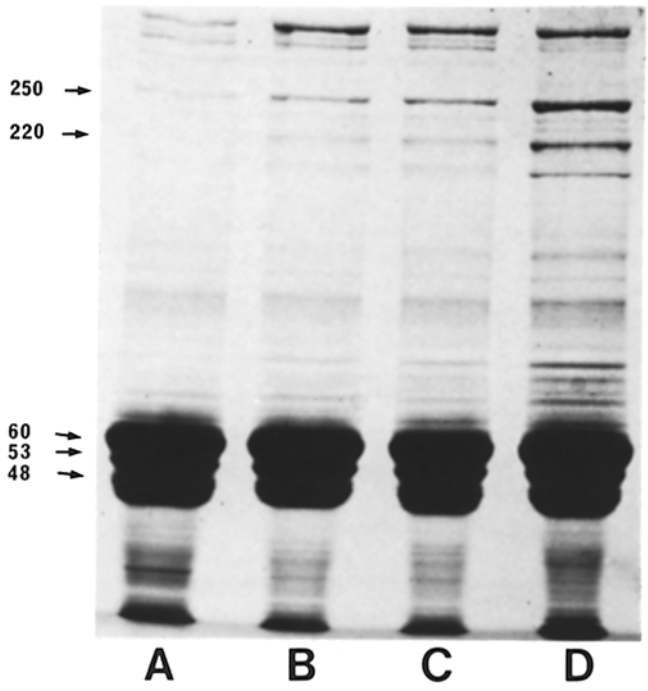

FIGURE 9 Cytoskeletons prepared from keratinocytes before the induction of desmosome formation (lane *A*) and at 2 h (lane *B*), 4 h (lane *C*), or 24 h (lane *D*) after the Ca^{2+} switch were analyzed by SDS PAGE. All of the preparations consists of three major polypeptides of 60, 53, and 48 kD. However, there are differences in the minor polypeptides in these preparations particularly in the high molecular weight range. A 220-kD protein is present in all preparations but appears maximally enriched 24 h after the initiation of desmosome formation (lanes *A* and *D*). A 220-kD polypeptide appears only in those preparations 2 h or more after the Ca^{2+} switch (lanes *B*, *C*, and *D*). Each lane contains ~20 μg of protein (estimated according to the procedure of Bradford [18]).

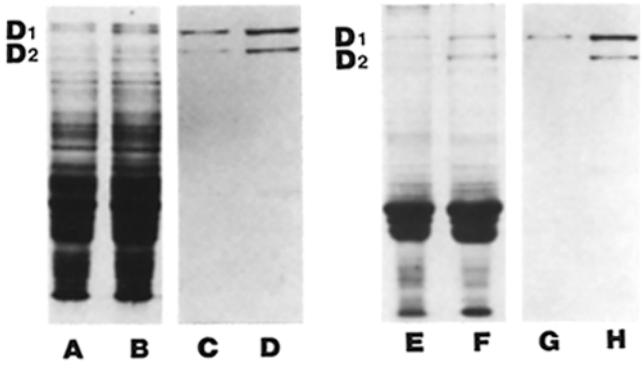

FIGURE 10 Whole PME cell protein extracts (30 μg of protein per lane) both before (lanes *A* and *C*) and 24 h after the induction of desmosome formation (lanes *B* and *D*) were subjected to SDS PAGE and transferred to nitrocellulose. Lanes *A* and *B* show amido black stains of the transferred proteins. Lanes *C* and *D* show immunoblots using the desmoplakin antiserum. Two polypeptides of 220 and 250 kD (desmoplakins 1 and 2 [*D1* and *D2*]) are recognized by this antiserum in both samples (lanes *C* and *D*). Cytoskeletal preparations (20 μg of protein per lane) isolated from PME cells maintained in LCa^{2+} (lanes *E* and *G*) and 24 h after the Ca^{2+} switch (lanes *F* and *H*) were also transferred to nitrocellulose. Lanes *E* and *F* show amido black stains of transferred proteins. Lanes *G* and *H* show the immunoblot with the desmoplakin antiserum. The desmoplakin antiserum recognizes a polypeptide of 250 kD in both samples (lanes *G* and *H*; desmoplakin 1 [*D1*]). However, this serum also recognizes a 220-kD (desmoplakin 2 [*D2*]) polypeptide found only in the cytoskeletal preparation isolated from PME cells in which desmosome formation has been induced (lane *H*).

ment (i.e., appropriate Ca^{2+} levels and cell–cell contact formation), desmoplakin is redistributed in the cytoplasm as desmosome morphogenesis progresses. Indeed, it would appear that all the components of desmosomes are preformed in PME cells before desmosome formation and are available to take part in the morphogenesis of these intercellular junctions, since Hennings and Holbrook (8) have shown that desmosomes form in PME cells in response to Ca^{2+} even in the presence of protein synthesis inhibitors.

Our preliminary biochemical observations appear to suggest that both desmoplakins 1 and 2 are present in the cytoplasm of PME cells that lack desmosomes (i.e., in LCa^{2+}). However, desmoplakin 1 appears to be associated with cytoskeleton preparations before the induction of desmosome morphogenesis, whereas desmoplakin 2 is only detected in the cytoskeleton preparations of PME cells in which desmosome formation has been induced. One possible explanation for these results is that both desmoplakin 1 and 2 are preformed in the cytoplasm of PME cells in LCa^{2+} but exhibit differences in detergent, high salt extractability. It is also possible that desmoplakin 2 is a product of desmoplakin 1, perhaps due to proteolytic cleavage. We cannot rule out the possibility that in our whole cell extracts of PME cells in LCa^{2+}, the polypeptide that has the same electrophoretic mobility as does desmoplakin 2 and is recognized by the desmoplakin antiserum is a proteolytic product of desmoplakin 1. The exact metabolic relationship of desmoplakins 1 and 2, two polypeptides that are extremely closely related (10), remains to be determined.

The coincident arrangement of IFB and desmoplakin-containing bodies, and the fact that desmoplakin 1 is associated with intermediate filament networks isolated both from cells maintained in LCa^{2+} and from those switched to NCa^{2+}, lead us to propose that desmoplakin 1 is an intermediate filament–associated protein in cultured mouse keratinocytes. Ultrastructural localization studies using desmoplakin antisera reveal that desmoplakin is localized at or near the region of the desmosome where IFB associate with the desmosomal plaque (reference 9; see also Fig. 7, *b* and *c*). We therefore speculate that desmoplakin 1 acts to initiate the association of IFB with the plasma membrane and also engages in desmosomal plaque formation.

Based on these findings, we would like to propose a model for desmosome formation in cultured keratinocytes. In PME cells, packages of desmosomal precursors (e.g., desmoplakin) are moved along with their associated IFB to sites of cell–cell contact upon addition of exogenous Ca^{2+}. These packages then appear to aggregate to form desmosomes. In this model, the association of the desmosome precursor–IFB complex with the plasma membrane appears to act as an initiator of desmosome formation, i.e., the desmosome does not act as a polymerization or initiation site for elaboration of IFB, at least in this cell culture system. We further presume that there are transmembranous or extracellular components that are involved in the alignment of the half desmosome precursor structures present in adjacent cells. When two such sets of extracellular components come into contact, they bind to each other, and desmosome formation can be completed. The possible mobility of desmosome components has been sug-

gested by others (21).

Although the mouse keratinocyte cell culture system (6) is proving to be very useful for studying the temporal sequence of desmosome formation under reasonably controlled cell culture conditions, we do not know whether the same sequence occurs in vivo. However, this system is advantageous for the analysis of the association of IFB with the plasma membrane (i.e., with the desmosomal junction). In this regard, we have initiated in vitro reconstitution experiments using purified preparations of desmoplakin and keratin to determine the biochemical characteristics of the IFB/desmoplakin association. Finally, we are in the process of studying the possible mechanisms underlying the movement and reorganization of both IFB and desmoplakins toward the cell membrane. It will be of interest to determine whether or not such movement requires the association of other cytoskeletal components such as microfilaments and microtubules.

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