Kinetics of Desmosome Assembly in Madin-Darby Canine Kidney Epithelial Cells: Temporal and Spatial Regulation of Desmoplakin Organization and Stabilization upon Cell-Cell Contact. II. Morphological Analysis

Manijeh Pasdar and W. James Nelson

Institute for Cancer Research, Philadelphia, Pennsylvania 19111

Abstract. Biochemical analysis of the kinetics of assembly of two cytoplasmic plaque proteins of the desmosome, desmoplakins I (250,000 M_r) and II (215,000 M_r), in Madin-Darby canine kidney (MDCK) epithelial cells, demonstrated that these proteins exist in a soluble and insoluble pool, as defined by their extract ability in a Triton X-100 high salt buffer (CSK buffer). Upon cell-cell contact, there is a rapid increase in the capacity of the insoluble pool at the expense of the soluble pool; subsequently, the insoluble pool is stabilized, while proteins remaining in the soluble pool continue to be degraded rapidly (Pasdar, M., and W. J. Nelson. 1988. *J. Cell Biol.* 106:677-685). In this paper, we have sought to determine the spatial distribution of the soluble and insoluble pools of desmoplakins I and II, and their organization in the absence and presence of cell-cell contact by using differential extraction procedures and indirect immunofluorescence microscopy. In the absence of cell-cell contact, two morphologically and spatially distinct patterns of staining of desmoplakins I and II were observed: a pattern of discrete spots in the cytoplasm and perinuclear region, which is insoluble in CSK buffer; and a pattern of diffuse perinuclear staining, which is soluble in CSK buffer, but which is preserved when cells are fixed in 100% methanol at −20°C. Upon cell-cell contact, in the absence or presence of protein synthesis, the punctate staining pattern of desmoplakins I and II is cleared rapidly and efficiently from the cytoplasm to the plasma membrane in areas of cell-cell contact (<180 min). The distribution of the diffuse perinuclear staining pattern remains relatively unchanged and becomes the principal form of desmoplakins I and II in the cytoplasm 180 min after induction of cell-cell contact. Thereafter, the relative intensity of staining of the diffuse pattern gradually diminishes and is completely absent 2-3 d after induction of cell-cell contact. Significantly, double immunofluorescence shows that during desmosome assembly on the plasma membrane both staining patterns coincide with a subpopulation of cytokeratin intermediate filaments. Taken together with the preceding biochemical analysis, we suggest that the assembly of desmoplakins I and II in MDCK epithelial cells is regulated at three discrete stages during the formation of desmosomes.

DESMOSOMES are major components of the junctional complex of epithelial cells and appear to be involved in maintaining the structural and functional interaction of adjacent cells (2-7, 10-13, 17, 18, 21, 29, 32). Electron microscopic studies of reaggregating cultures of epithelial cells have demonstrated that an early event in desmosome assembly is the appearance of symmetrical cytoplasmic plaques on the plasma membranes of adjacent cells (26-28). These plaques contain two major proteins, desmoplakins I (250,000 M_r) and II (215,000 M_r) (8, 21, 33). In the absence of cell-cell contact, desmoplakins I and II are located in the cytoplasm as discrete spots that reorganize onto the plasma membrane upon cell-cell contact (15, 34). These studies raise several questions concerning the regulation of assembly of desmoplakins I and II upon cell-cell contact, and the nature of the reorganization of these cytoplasmic spots of desmoplakins I and II.

Our biochemical analysis of the kinetics of assembly of desmoplakins I and II in Madin-Darby canine kidney (MDCK) cells provided new insights into these events (see accompanying paper, reference 30). Our study showed that upon cell-cell contact, desmoplakins I and II are recruited as a complex from a soluble pool into an insoluble pool of protein, which is subsequently stabilized. These results suggest that the soluble pool of desmoplakins I and II is a precursor to the insoluble pool, and that the latter can be rapidly assembled into desmosomes upon cell-cell contact.

In the context of this new biochemical analysis, we have sought to determine the spatial organization of the soluble and insoluble intracellular pools of desmoplakins I and II in

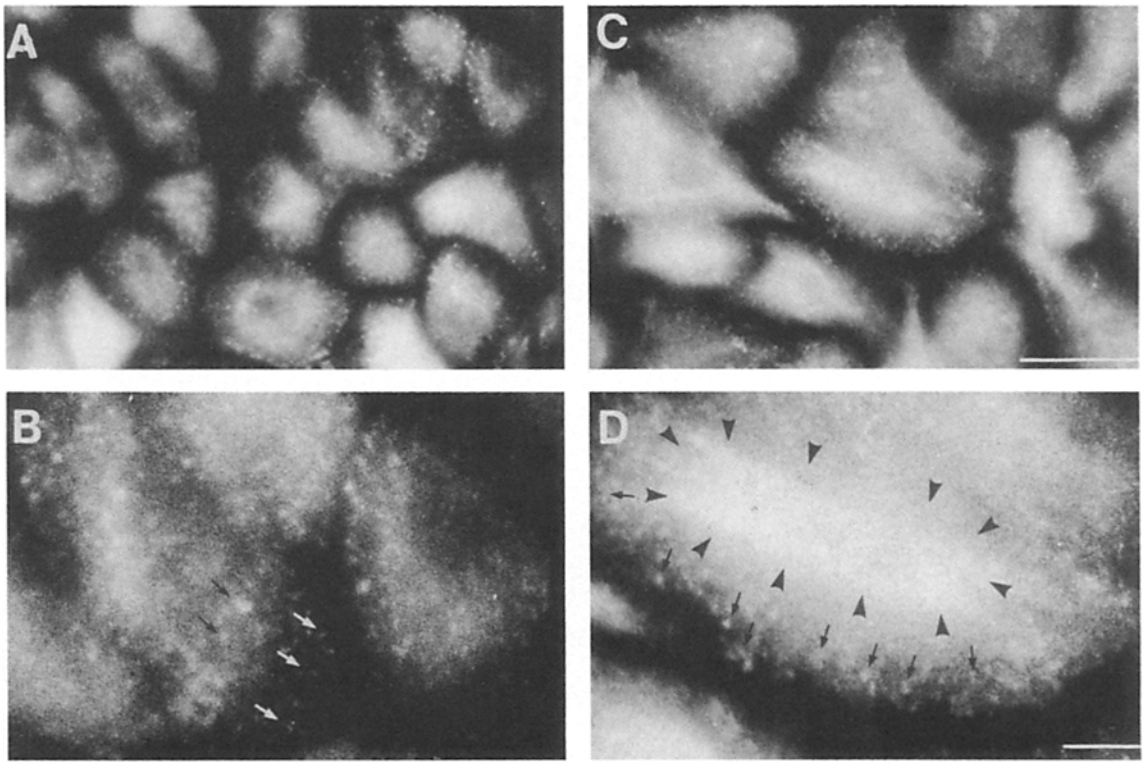


Figure 1. Indirect immunofluorescence of desmoplakins I and II in MDCK cells in the absence of cell–cell contact. Confluent monolayers of MDCK cells were established in LC medium for 12 h. Cells were extracted in situ with CSK buffer and fixed with 1.75% wt/vol formaldehyde in PBS (*A* and *B*), or fixed and permeabilized in 100% methanol at −20°C (*C* and *D*). Cells were processed for indirect immunofluorescence with a 1/100 dilution of antiserum for desmoplakins I and II followed by affinity-purified biotinylated goat anti-rabbit IgG and avidin-conjugated FITC. *B* and *D* are higher magnifications of cells in *A* and *C*, respectively. Cells extracted in CSK buffer (*A* and *B*) exhibit a cytoplasmic punctate staining pattern (*arrows*), whereas cells fixed in 100% methanol at −20°C (*C* and *D*) exhibit the punctate pattern (*arrows*) superimposed on a diffuse perinuclear staining pattern (*arrowheads*). Bars: (*A* and *C*) 20 μm; (*B* and *D*) 5 μm.

MDCK epithelial cells. We show that in the absence of cell–cell contact, desmoplakins I and II are located in two morphologically and spatially distinct intracellular pools of protein: a punctate pattern, which is insoluble in cytoskeleton extraction buffer (CSK) buffer,[1] and a diffuse pattern, which is soluble in CSK buffer. Upon cell–cell contact, in the absence or presence of protein synthesis, there is a rapid and selective reorganization of only the punctate staining pattern from the cytoplasm to the plasma membrane in areas of cell–cell contact. Significantly, the punctate and diffuse staining patterns appear to overlap along cytokeratin intermediate filaments. These results are discussed together with the biochemical analysis in the context of the regulation of assembly of desmoplakins I and II.

1. *Abbreviations used in this paper*: CSK buffer, cytoskeleton extraction buffer; HC medium, high Ca^{++} (1.8 mM) medium; LC medium, low Ca^{++} (5 μM) medium.

Materials and Methods

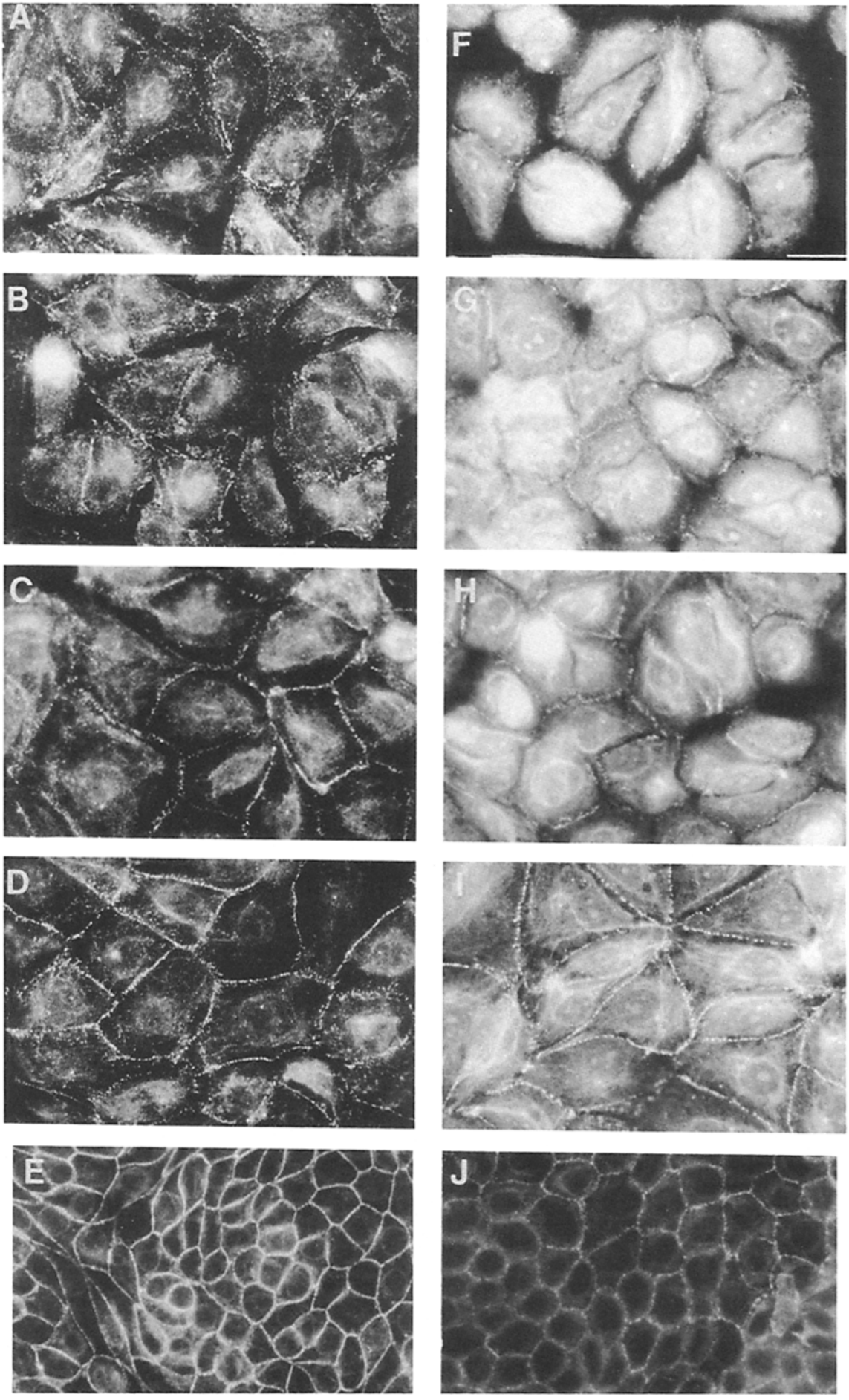
Cells

The experimental conditions for establishing and maintaining cultures of confluent MDCK cells in low Ca^{++} (5 μM) (LC) or high Ca^{++} (1.8 mM) (HC) medium have been described in detail previously (22; see also reference 30).

Antibodies

The antibodies for desmoplakins I and II were raised against bovine epidermal desmoplakin II (215,000 M_r) and have been characterized extensively in the preceding paper (30). By immunofluorescence, the antibody stained discrete spots on the plasma membrane of cells in confluent monolayers characteristic of the distribution of desmosomes (see Fig. 2). Significantly, absorption of the antiserum with either purified desmoplakin I and II resulted in a loss of this staining pattern, indicating that the staining was specific (data not shown). A mouse monoclonal antibody specific for human epithelial cytokeratins (AE1:AE3), which reacts with MDCK cell cytokeratins, was purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN.

Figure 2. Indirect immunofluorescence of desmoplakins I and II in MDCK cells during induction of cell–cell contact. Confluent monolayers of MDCK cells were established in LC medium for 12 h. Cells were then cultured in HC medium for 15 (*A* and *E*), 30 (*B* and *F*), 60 (*C* and *G*), 180 min (*D* and *H*) or 3 d (*E* and *J*). Cells were extracted with CSK buffer and fixed with 1.75% wt/vol formaldehyde in PBS (*A–E*), or fixed and permeabilized in 100% methanol at −20°C (*F–J*). Cells were processed for indirect immunofluorescence with a 1/100 dilution of antiserum of desmoplakins I and II, affinity-purified biotinylated goat anti-rabbit IgG, and avidin-conjugated FITC. Bars: (*A–D* and *F–H*) 20 μm; (*E* and *J*) 40 μm.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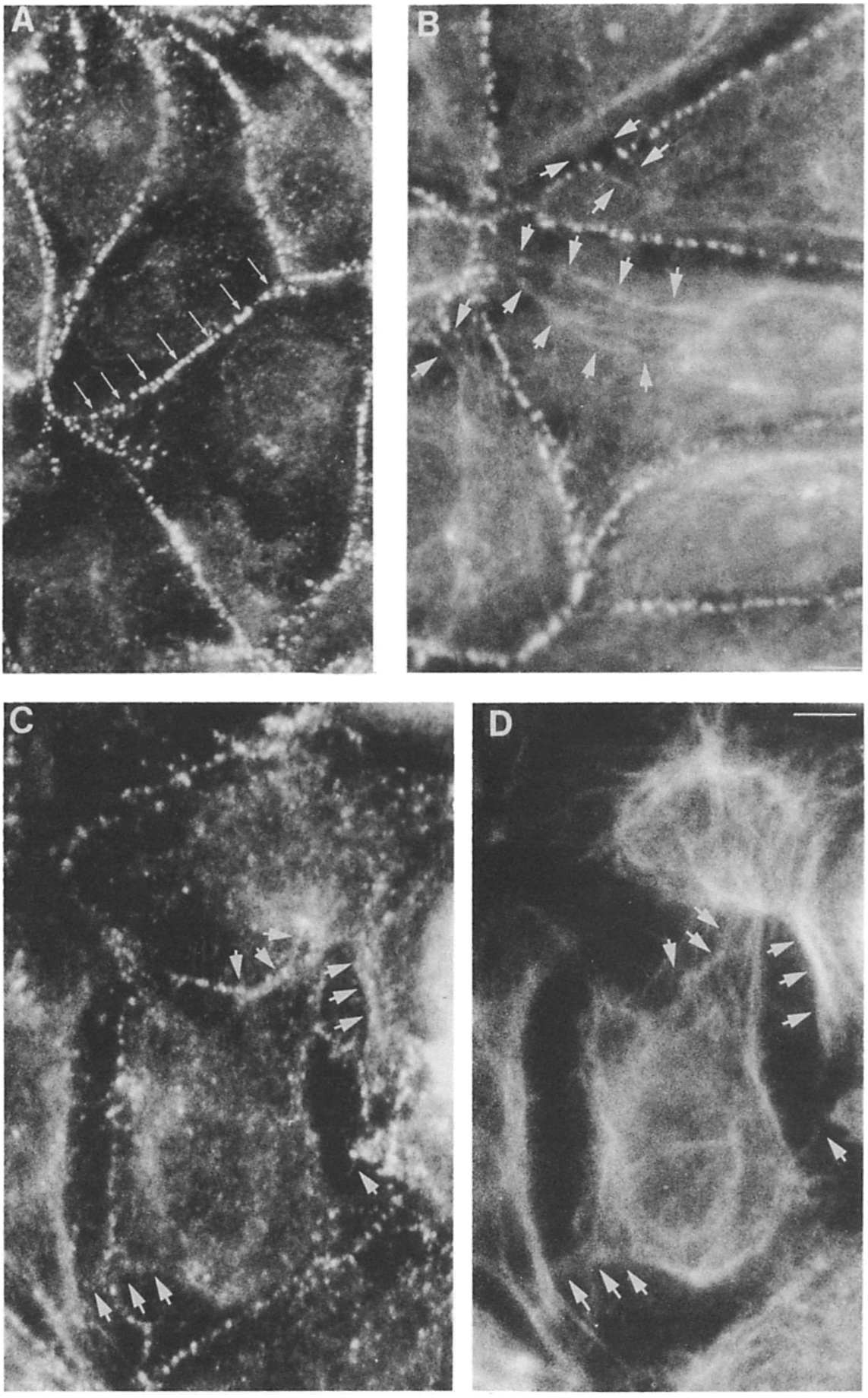
A
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Indirect Immunofluorescence Microscopy

Replicate cultures of MDCK cells grown on collagen-coated coverslips were rinsed briefly in PBS and then processed for indirect immunofluorescence in one of three ways: (*a*) cells were fixed in 100% methanol at −20°C for 5–10 min; (*b*) cells were permeabilized with 0.5% vol/vol Triton X-100 (10–15 min) in PBS, and then fixed in 1.75% wt/vol formaldehyde in PBS for 5–10 min at room temperature; (*c*) cells were extracted with CSK buffer (see preceding paper for details; reference 30) and then fixed in 1.75% wt/vol formaldehyde for 5–10 min at room temperature. After the fixation step, all cells were treated identically. Cells were washed in PBS and incubated with a 1:100 dilution of the desmoplakins I and II antibodies in PBS for 1 h at 37°C in a humidified atmosphere. For double labeling, a 1:50 dilution of a mouse monoclonal antibody specific for epithelial cytokeratins in PBS was applied together with the rabbit polyclonal antibodies specific for desmoplakins I and II. Cells were washed extensively in PBS, and were then incubated with a 1:50 dilution of affinity-purified biotinylated goat anti-rabbit IgG (Boehringer-Mannheim Biochemicals) in PBS for 30 min as above. The cells were washed in PBS and then incubated with a 1:50 dilution of avidin-conjugated FITC (Boehringer-Mannheim Biochemicals) in PBS for 30 min as above. Cells were washed in PBS, and those processed for double labeling were incubated with a 1/50 dilution of affinity-purified goat anti-mouse IgG conjugated with rhodamine (Boehringer-Mannheim Biochemicals) in PBS for 30 min as above. After extensive washing in PBS, coverslips were mounted in Elvanol (31) and viewed with a 63× objective using a Zeiss Universal microscope equipped with epifluorescence illumination. Immunofluorescence images were photographed on Tri-X pan film, and all negatives and positives were taken with approximately the same exposure to facilitate comparisons.

Results

Distribution of Desmoplakins I and II in Confluent Monolayers of MDCK Cells in the Absence of Cell–Cell Contact

When MDCK cells grown in LC medium were extracted with CSK buffer before fixation in 1.75% wt/vol formaldehyde (Fig. 1, *A* and *B*), a punctate pattern of staining of desmoplakins I and II was observed throughout the cytoplasm (Fig. 1 *B*; see also Fig. 2, *A–F*). In a minority of cells, this pattern was more concentrated in the perinuclear region, giving the appearance of a more intense uniform staining pattern (Fig. 1 *A*). However, higher magnification revealed that there was a predominance of fluorescent spots in these regions identical in morphology to those in the rest of the cytoplasm (Fig. 1 *B*).

When cells were fixed in 100% methanol at −20°C we detected two fluorescent staining patterns with desmoplakins I and II antiserum (Fig. 1, *C* and *D*; see also Fig. 2, *F–J*). First, there was a distinct punctate pattern in the cytoplasm similar to that found after extraction of cells in CSK buffer (see above). Second, there was a diffuse cytoplasmic fluorescence which appeared to be relatively more concentrated in the center of the cells than at the periphery and exhibited little or no distinctive morphology at higher magnification (Fig. 1 *D*).

Selective Reorganization of Desmoplakins I and II upon Induction of Cell–Cell Contact

Confluent monolayers of MDCK cells were established in LC medium for ~12 h, at which time the medium was replaced by HC-medium in order to synchronously induce cell–cell contact. At all times after induction of cell–cell contact, cells extracted in CSK buffer exhibited predominantly, if not exclusively, a discrete punctate pattern of staining with desmoplakin I and II antiserums. However, there was a significant and complete reorganization of this staining pattern. As early as 15 (Fig. 2 *A*)–30 min (Fig. 2 *B*) after induction of cell–cell contact, distinct lines of punctate fluorescent staining were observed at the plasma membrane in areas of cell–cell contact. Subsequently, at 60 (Fig. 2 *C*) and 180 min (Fig. 2 *D*), the punctate fluorescent staining at the plasma membrane increased dramatically. Concomitantly, the punctate pattern of staining within the cytoplasm gradually decreased, such that at 180 min the cytoplasm exhibited little or no fluorescent spots (Fig. 2 *D*). This staining pattern was identical to that observed 3 d after cell–cell contact (Fig. 2 *E*).

Confluent monolayers of MDCK cells fixed in 100% methanol at −20°C before immunofluorescence revealed that the two patterns of fluorescent staining underwent a differential reorganization upon cell–cell contact. The punctate cytoplasmic pattern of staining was rapidly cleared from the cytoplasm to the plasma membrane in regions of cell–cell contact with kinetics similar to that observed after extraction with CSK buffer (see above). The diffuse staining pattern exhibited a less marked reorganization. At time points up to 180 min after induction of cell–cell contact (Fig. 3, *F–I*), the diffuse perinuclear and cytoplasmic staining was very prominent in all cells. However, in time, this diffuse cytoplasmic pattern of staining diminished in intensity, such that at 3 d after induction of cell–cell contact little or none remained (Fig. 2 *J*).

Although the two staining patterns identified by extraction of cells in 100% methanol at −20°C exhibited a differential reorganization upon cell–cell contact, observations at higher magnification revealed that the patterns were interconnected (Fig. 3 *B*). The diffuse staining pattern appeared to consist of distinctive strands in the cytoplasm that were directly connected to discrete fluorescent spots on the plasma membrane in the regions of cell–cell contact. Occasionally, fluorescent spots appeared to be localized on these strands close to, but not on the plasma membrane (Fig. 3 *B*). Parallel staining of cells extracted in CSK buffer revealed only the punctate pattern of desmoplakins I and II, and no evidence of these distinctive strands (Fig. 3 *A*); thus, this material is defined as comprising CSK buffer-soluble desmoplakins I and II. Significantly, double immunofluorescence microscopy with

Figure 3. Indirect immunofluorescence of desmoplakins I and II in MDCK cells during induction of cell–cell contact; high magnification. Confluent monolayers of MDCK cells were established in LC medium and then induced to form cell–cell contact in HC medium. 3 h after induction of cell–cell contact, cells were either extracted with CSK buffer and fixed with 1.75% wt/vol formaldehyde in PBS (*A*, *C*, and *D*), or fixed and permeabilized with 100% methanol at −20°C (*B*). Cells were processed for indirect immunofluorescence with a 1/100 dilution of antiserum of desmoplakins I and II followed by affinity-purified goat anti-rabbit IgG and avidin-conjugated FITC. Alternatively, cells were processed for double immunofluorescence with a 1/100 dilution of antiserum of desmoplakins I and II (*C*) and a 1/50 dilution of a monoclonal antibody specific for cytokeratin intermediate filaments (*D*), followed by affinity-purified biotinylated goat anti-rabbit IgG and avidin-conjugated FITC (*A* and *B*), and rhodamine-labeled goat anti-mouse IgG (*C* and *D*). Arrows indicate areas of codistribution of cytokeratin intermediate filaments and desmoplakins I and II. Bar, 5 μm.

antibodies specific for desmoplakins I and II and cytokeratin intermediate filament proteins showed that these distinctive strands of desmoplakins I and II staining coincided with the distribution of individual strands of cytokeratin intermediate filaments (see Fig. 3, *C* and *B*).

Together, these results demonstrate that there is a rapid and selective reorganization of desmoplakins I and II upon induction of cell-cell contact. The CSK buffer-insoluble fraction, represented by the punctate pattern of staining, is cleared rapidly from the cytoplasm to the plasma membrane in regions of cell-cell contact. On the other hand, the CSK buffer-soluble fraction, represented by the diffuse cytoplasmic pattern of staining observed in methanol-fixed cells, undergoes little redistribution upon induction of cell-cell contact. However, the results indicate that close to the plasma membrane the punctate and diffuse staining patterns of desmoplakins I and II are directly interconnected along cytoplasmic strands that appear to represent cytokeratin intermediate filaments

Discussion

Previous studies of the spatial organization of desmosomal proteins in MDCK cells have extensively documented the presence of a punctate staining pattern of desmoplakins I and II before and after induction of cell-cell contact (8, 19–21). Part of the present study confirms these previous results. However, the significance of our reevaluation of the subcellular distribution of desmoplakins I and II is that we have been able to identify two spatially distinct pools of protein in MDCK cells. These pools of protein undergo differential reorganization upon cell-cell contact, which correlates well with our biochemical analysis of the fates of an insoluble and soluble pools of desmoplakins I and II that were identified in the preceding paper. Therefore, taken together with the preceding biochemical analysis, this morphological study provides a new insight into the temporal and spatial regulation of assembly of the desmosome upon cell-cell contact.

Identification of Two Spatially Distinct Pools of Desmoplakins I and II in MDCK Cells

Examination of MDCK cells by indirect immunofluorescence after extraction with CSK buffer revealed that, in the absence of cell-cell contact, desmoplakins I and II were present in a pattern of discrete spots similar to that reported recently (1, 15, 19, 34). However, when cells were fixed in 100% methanol at −20°C and then processed for indirect immunofluorescence, we found a more complex staining pattern of desmoplakins I and II (Figs. 1 and 2). We observed a cytoplasmic staining pattern comprised of discrete spots similar in morphology, quantity, and distribution to that of the punctate pattern detected in cells after extraction with CSK buffer. Superimposed on this punctate pattern was a diffuse cytoplasmic staining pattern. The fact that this diffuse staining pattern was not detected when cells were extracted with CSK buffer before immunofluorescence indicates that it represents a CSK buffer-soluble pool of desmoplakins I and II. On the other hand, the discrete punctate staining pattern was observed after either of the extraction/fixation protocols, indicating that it represents a CSK buffer-insoluble pool of desmoplakins I and II. Thus, our morphological analysis of desmoplakins I and II has identified two pools of protein in MDCK cells that appear to correspond to the CSK buffer-soluble and -insoluble pools determined in our preceding biochemical analysis.

It is significant that these staining patterns were present in MDCK cells that had not had cell-cell contact for 48–60 h (see Materials and Methods and reference 23). Since the CSK buffer-soluble and -insoluble pools of desmoplakins I and II are relatively unstable in these cells ($t_{½} \sim$ 8–10 h; reference 30), it is probable that structures remaining from previous disassembled desmosomes would have been completely degraded within this time period (the total period of time that the cells were cultured in the absence of cell-cell contact was equivalent to 5–7 half-lives of the proteins). In fact, we found that after loss of cell-cell contact, desmoplakins I and II were degraded rapidly ($t_{½} \sim$ 8 h; data not shown). Therefore, in the absence of cell-cell contact, both pools of protein are continuously turned over and replenished with newly synthesized proteins (see below).

Temporal and Spatial Coordination between Cell-Cell Contact and the Differential Reorganization of Pools of Desmoplakins I and II

Upon induction of cell-cell contact we found that the spatial distribution of the two pools of desmoplakins I and II underwent differential reorganization. After either extraction of cells in CSK buffer or fixation in 100% methanol at −20°C, we observed that the punctate staining pattern was cleared rapidly from the cytoplasm to the plasma membrane in the regions of cell-cell contact, as has been reported previously (13, 15, 24, 26, 34). On the other hand, we detected little or no change in the distribution of the diffuse staining pattern, which remained in the perinuclear region and in the cytoplasm. However, we noticed that the intensity of staining of the diffuse pattern gradually diminished, such that 3 d after induction of cell-cell contact little or no diffuse staining was detected in the cytoplasm.

The differential reorganization of these two pools of desmoplakins I and II upon cell-cell contact correlates with changes in the capacity and stability of the CSK buffer-insoluble and -soluble pools of protein determined in our biochemical analysis of desmoplakins I and II (reference 30). First, the rapid movement of the punctate staining pattern from the cytoplasm to the plasma membrane correlates with a dramatic increase in the capacity and stability of the insoluble pool of desmoplakins I and II. This coordinate stabilization and reorganization of the insoluble pool of desmoplakins I and II may reflect the assembly of these proteins into desmosomes on the plasma membrane. Indeed, previous electron microscope studies of desmosome formation in reaggregated cultures of epithelial cells have shown that the cytoplasmic plaques of desmosomes, which contain desmoplakins I and II, form rapidly at the plasma membrane between 15–180 min after induction of cell-cell contact (14, 15, 19, 24–28, 29).

Significantly, we detected little or no effect of cell-cell contact on the stability ($t_{½} \sim$ 8–10 h; reference 30) or the spatial organization of the CSK buffer-soluble pool of desmoplakins I and II (diffuse staining pattern). Consistent with the rapid degradation of this pool of proteins, we noticed that the diffuse staining pattern gradually disappeared after in-

duction of cell–cell contact (Fig. 2). We suggest that the disappearance of this particular staining pattern was due to an initial titration of proteins into the insoluble pool (see below), followed by the gradual proteolytic degradation of the remainder.

Linkage of the Soluble and Insoluble Pools of Desmoplakins I and II by Cytokeratin Intermediate Filaments

Our biochemical analysis of the assembly of desmoplakins I and II revealed that induction of cell–cell contact resulted in a rapid increase in the capacity of the insoluble pool of proteins. That there was a concomitant decline in the amount of protein in the soluble pool indicated that proteins were being recruited directly from the soluble pool into the insoluble pool. Significantly, our analysis of the spatial organization of desmoplakins I and II by immunofluorescence revealed an apparent interconnection of the CSK buffer-soluble and -insoluble pools of protein. High magnification of MDCK cells that had been fixed in 100% methanol at −20°C, 180 min after induction of cell–cell contact (Fig. 3 *A*) revealed that the punctate staining pattern (CSK buffer–insoluble pool) was almost exclusively localized to the plasma membrane of adjacent cells. In the same cells, distinctively stained strands emanating from the more intensely staining perinuclear region (CSK buffer–soluble pool) appeared to traverse the cytoplasm and contact directly with individual fluorescent spots on the plasma membrane (Fig. 3 *B*).

How are the soluble and insoluble pools of desmoplakins I and II interconnected? Cytokeratin intermediate filaments are known to be associated with the cytoplasmic plaque of assembled desmosomes (5, 7, 9, 15, 21). Double immunofluorescence of MDCK cells extracted with CSK buffer revealed a distinct interconnection of intermediate filaments and the punctate staining pattern of desmoplakins I and II (Fig. 3, *C* and *D*; see also, references 1, 5, 13, 15, 19, 20). Double immunofluorescence after fixation of MDCK cells in 100% methanol revealed that individual bundles of cytokeratin intermediate filaments, which were connected to the fluorescent spots of desmoplakins I and II, were codistributed with the diffuse staining pattern of these desmosomal proteins (data not shown).

That cytokeratin intermediate filaments may play a role in the assembly of desmosomes has been suggested previously. Hennings and Holbrook (13), Jones and Goldman (15), and Jones et al. (16) have postulated that the reorganization of cytokeratin intermediate filaments in keratinocytes is the initial event in the assembly of desmosomes. However, Bologna et al. (1) suggested that desmosome formation was a prerequisite for the organization of the intermediate filaments in rat mammary epithelial cells. Our results support the view that cytokeratin intermediate filaments may function as a nucleation site for the assembly of insoluble complexes of desmoplakins I and II in the cytoplasm (15). We suggest that the association of the soluble (diffuse staining pattern) and insoluble (punctate pattern) pools of desmoplakins I and II with cytokeratin intermediate filaments may allow the rapid titration of complexes of desmoplakins I and II from the soluble pool directly into the insoluble pool. Upon induction of cell–cell contact, these insoluble complexes of desmoplakins I and II move rapidly from the cytoplasm to the plasma membrane in association with or along individual bundles of intermediate filaments, and become organized into desmosomes in regions of cell–cell contact by elements of the desmosomal membrane core. We suggest that the nucleation of desmoplakins I and II into preformed, insoluble complexes on cytokeratin intermediate filaments in the cytoplasm may be functionally important, since it would enable the cell to respond rapidly to cell–cell contact through the fast assembly of desmosomes at the plasma membrane, which, in turn, would lead to the maintenance of appropriate cell–cell interactions. At present, we do not know the mechanism(s) of desmoplakin association with cytokeratin intermediate filaments. However, the requirement of cytokeratin intermediate filaments for the nucleation of assembly of desmoplakins I and II could be tested directly by analyzing the effect of disruption of cytokeratin intermediate filaments on the organization and stability of desmoplakins I and II.

Temporal and Spatial Regulation of Assembly of Desmoplakins I and II upon Cell–Cell Contact in MDCK Epithelial Cells

These biochemical and morphological analyses of organization of desmoplakins I and II upon cell–cell contact have identified at least three stages in the assembly of these proteins from soluble subunits into desmosomes (see Fig. 4). We propose that stage I in assembly involves the formation of a soluble complex of desmoplakins I and II in a ratio of ~3–4:1. This soluble pool of protein exhibits a diffuse staining pattern that is colocalized with, but not bound tightly to, cytokeratin intermediate filaments in the cytoplasm and perinuclear region of the cell. Stage II involves the nucleation of these soluble protein complexes into an insoluble pool of protein that becomes tightly bound to the cytokeratin intermediate filaments; this insoluble pool of desmoplakins I and II exhibits a punctate staining pattern in the cytoplasm. Stages I and II occur in the presence or absence of cell–cell contact, but, in the absence of cell–cell contact, both pools of proteins are degraded rapidly ($t_{1/2} \sim$ 8–10 h).

Upon cell–cell contact, the fate of the soluble and insoluble pools of desmoplakins I and II is dramatically different (stage III). The capacity of the insoluble pool increases threefold as protein complexes are recruited rapidly from the soluble pool into the insoluble pool. This recruitment lasts for 3–6 h after the induction of cell–cell contact. During this time, the CSK buffer–insoluble pool of desmoplakins I and II complexes (punctate pattern) moves rapidly from the cytoplasm to the plasma membrane in regions of cell–cell contact. These insoluble protein complexes, now part of assembled desmosomes on the plasma membrane, are stabilized ($t_{1/2} >$ 72 h), whereas protein complexes remaining in the soluble pool are rapidly degraded ($t_{1/2} \sim$ 8–10 h). The majority of the cytokeratin intermediate filament network then becomes reorganized at the cell periphery in association with the desmosomes. The remaining desmoplakins I and II in the soluble pool are degraded, resulting in the gradual loss of the diffuse staining pattern in the cytoplasm.

What factor(s) regulates the transition of complexes of desmoplakins I and II between these three proposed stages in the assembly of desmosomes? Experiments using cycloheximide to inhibit protein synthesis at the time of induction of cell–cell contact suggest that proteins synthesized coor-

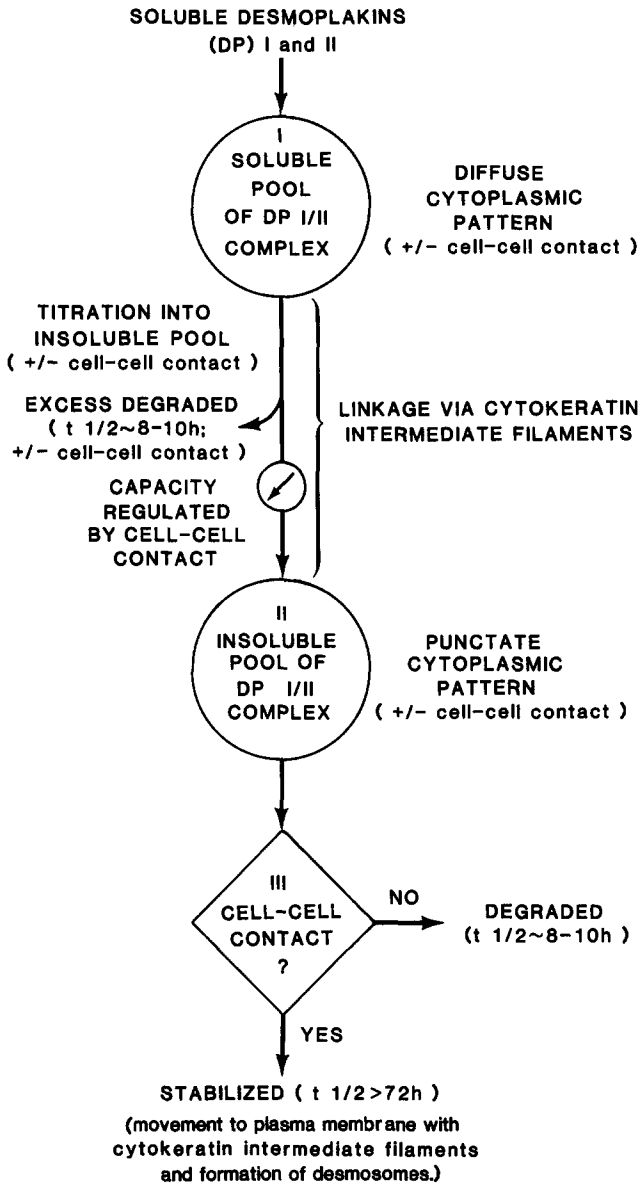

Figure 4. Stages in the temporal and spatial regulation of stabilization and assembly of desmoplakins I and II in MDCK epithelial cells. For details see text and preceding paper (30). *DP*, desmoplakin.

dinately with desmoplakins I and II regulate their stabilization and accumulation. However, desmoplakins I and II that had already accumulated at steady state together with other protein(s) were assembled rapidly into desmosomes in the presence of cycloheximide and cell–cell contact (13; Pasdar, M., and W. J. Nelson, unpublished results). Since the desmosome is a multisubunit complex comprised of integral membrane proteins and extracellular adhesion proteins (2, 6, 7, 18, 33), it is possible that at least one of these proteins regulates the stabilization and assembly of desmoplakins I and II. We are currently analyzing the effects of biosynthesis and organization of these proteins upon cell–cell contact to test this possibility.

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