

# Calcium-induced Reorganization of Desmosomal Components in Cultured Human Keratinocytes

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**ABSTRACT** We used antibodies raised against individual desmosomal components to study calcium-induced desmosome formation in human keratinocytes. When keratinocytes are forced to grow as a monolayer by reducing the level of calcium ions in the culture medium, there is little contact between adjacent cells. Raising the level of calcium ions rapidly induces desmosome formation, and stratification occurs within 24 h. We found that before addition of calcium the 115,000- and 100,000-mol-wt core glycoproteins were distributed over the entire cell surface, whereas the plaque proteins (205,000 and 230,000 mol wt), the 82,000- and 86,000-mol-wt proteins, and the 150,000-mol-wt glycoprotein were located throughout the cytoplasm. 15 min after increasing the calcium ion concentration, all of these molecules appeared at the cell margins. The intensity of peripheral staining increased over the next 2 h and during this time the distribution of keratin filaments changed from predominantly perinuclear to extend throughout the cytoplasm. Keratinocytes could be dissociated with EDTA for up to 2 h after exposure to calcium. After 3 h of exposure to calcium the cells were no longer susceptible to EDTA dissociation and staining for desmosomal plaque antigens persisted in regions of intercellular contact. Desmosomal staining in stratified cultures became greatly reduced within 24 h of lowering the calcium ion concentration again. We have preliminary evidence that stratification occurs by breakdown of desmosomes at lateral surfaces and reformation at surfaces of contact between basal and suprabasal cells, rather than by rearrangement of existing desmosomes. Involucrin-positive cells in the monolayer appeared to contain more 205,000- and 230,000-mol-wt proteins free in the cytoplasm than involucrin-negative cells.

Desmosomes are junctions that hold epithelial cells together; they are most abundant in tissues, such as epidermis, that are subject to mechanical stress. Despite many ultrastructural studies, there is no general agreement on the precise timing and sequence of events in desmosome formation (7, 13, 15, 17, 21, 22, 24, 25). Electron microscopy has the limitations that it cannot provide information about changes preceding the formation of morphologically distinct structures, and it does not give an overview of desmosome assembly in whole cell populations. The recent availability of antibodies to individual desmosomal components (3, 4, 9, 18) now makes it possible to examine desmosome formation at the molecular level.

Polyclonal antisera against each of the five high molecular weight desmosomal proteins reveal the distribution of desmosomes in a range of tissues from different vertebrate species

(4, 5). Desmosomal plaques contain two proteins with molecular weights of 205,000 and 230,000, named desmoplakins by Franke and co-workers (8, 9); these proteins appear to be closely related and have common antigenic sites. Two other proteins of molecular weights 82,000 (basic pI) and 86,000 (neutral pI) (18) may also be in the plaque (11), although evidence for their precise localization is lacking. Three glycoproteins found in desmosomes have molecular weights of 150,000, 115,000, and 100,000 (11). The 115,000- and 100,000-mol-wt glycoproteins are found on the cell surface and have been named desmocollins I and II, because of their role in desmosomal adhesion (6).

Cultured keratinocytes provide a useful experimental system for the study of desmosome formation (13). When these cells are maintained in low-calcium medium ( $\leq 0.1$  mM) they proliferate, but do not stratify. Under these conditions there

is little cell-cell contact and the cells do not form desmosomes (14). Raising the level of calcium ions (to 2.0 mM) rapidly induces desmosome formation, and stratification occurs within 24 h. During stratification, there is selective migration of terminally differentiating cells from the basal layer (35).

In this paper, we show that raising the calcium concentration causes rapid and synchronous accumulation of all desmosomal components in regions of intercellular contact. Calcium also induced a dramatic reorganization of keratin filaments. We investigated the effect of EDTA on the organization of desmosomal components and examined the distribution of desmosomes in stratified cells.

## MATERIALS AND METHODS

**Cell Culture:** Human keratinocytes from newborn foreskin (strain a, third to seventh passage) were grown on glass coverslips with a feeder layer of mitomycin C-treated 3T3 cells (26, 27). Control cultures were grown in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum, 0.5  $\mu$ g/ml hydrocortisone (27),  $10^{-10}$  M cholera toxin (12), and 10 ng/ml epidermal growth factor (28). For low-calcium cultures, calcium salts were omitted from the medium formulation and fetal calf serum was depleted of divalent cations by treatment with Chelex 100 resin (Bio-Rad Laboratories, Richmond, CA), as described by Brennan et al. (2). The calcium concentration was  $\sim$ 2.0 mM in control medium, and 0.1 mM in low-calcium medium.

**Antibodies:** Individual desmosomal proteins from bovine nasal epithelium were separated by PAGE, eluted, and injected into guinea pigs (4). The resulting antisera were preabsorbed with bovine epidermal keratin to remove any contaminant keratin antibodies (9). The antisera are named according to the molecular weights ( $\times 10^{-3}$ ) of the desmosomal components against which they were raised: anti-205/230, anti-150, anti-115, anti-100, and anti-82/86. The specificity of these antibodies has been fully described previously (4). It should be noted that anti-115 and anti-100 each react with both the 115,000- and 100,000-mol-wt antigens.

LP34 is a monoclonal IgG<sub>1</sub> that binds to keratin filaments in all human epidermal keratinocytes (E. B. Lane, P. E. Purkis, and I. M. Leigh, manuscript in preparation). Rabbit antiserum to involucrin was prepared either as described previously (29) or by injecting involucrin eluted from polyacrylamide gels.

Rhodamine-conjugated goat anti-rabbit IgG and fluorescein-conjugated rabbit anti-mouse IgG were purchased from Miles Scientific (Naperville, IL). Fluorescein-conjugated sheep anti-guinea pig IgG was obtained from Wellcome Laboratories (Beckenham, England) and used with anti-205/230, anti-150, and anti-82/86. Biotinylated sheep anti-guinea pig IgG and rhodamine-conjugated avidin were supplied by Vector Laboratories, Inc. (Burlingame, CA) and used with anti-115 and anti-100.

**Immunofluorescence:** Keratinocytes were fixed for 5 min in absolute methanol on ice for staining with LP34, anti-205/230, anti-150, and anti-82/86. For anti-115 and anti-100, fixation was in 3.7% formaldehyde in PBS at room temperature for 8 min. In double label immunofluorescence experiments, cells were fixed in methanol and incubated successively with antiserum to involucrin, rhodamine-conjugated goat anti-rabbit IgG, normal rabbit serum, anti-205/230, and fluorescein-conjugated sheep anti-guinea pig IgG. All incubations of fixed cells with antisera were at room temperature for 20–30 min, and cells were washed extensively with PBS between incubations. Coverslips were mounted in Gelvatol (Monsanto Co., St. Louis, MO) and examined with a Zeiss photomicroscope III, using incident illumination.

## RESULTS

### Time Course of Desmosome Formation

Keratinocytes were fixed 0, 5, 10, 15, 30, 60, and 120 min after replacing low-calcium medium (0.1 mM) with normal medium (2.0 mM). In low-calcium medium, virtually all the cells lacked peripheral staining and the cytoplasm stained diffusely with anti-205/230 (Fig. 1 *a*). Identical staining was observed with anti-150 and anti-82/86. The 115,000- and 100,000-mol-wt glycoproteins were distributed over the entire cell surface as demonstrated by incubation with whole antiserum at 37°C for 30 min before fixation (Fig. 1 *b*). In low-calcium medium, most of the keratin filaments were concen-

trated round the nucleus and did not extend into the peripheral cytoplasm (Fig. 1 *c*).

15 min after increasing the calcium ion concentration, the cytoplasmic desmosomal components were distributed in a distinct punctate pattern at the cell margins (Fig. 1 *d*). The 100,000- and 115,000-mol-wt cell surface glycoproteins were localized mainly at the cell boundaries, although some staining was also seen over the rest of the cell. From 15 min to 2 h the pattern of staining remained essentially the same, but the general level of cytoplasmic fluorescence decreased and desmosomal staining at the cell periphery increased in intensity (Fig. 1, *e–h*). In some cells the distribution of cytoplasmic antigens was fibrillar in subperipheral regions (Fig. 1, *g* and *h*). The accumulation of desmosomal antigens at the cell periphery was thus synchronous, both with respect to individual cells and to individual desmosomal components.

There was a gradual rearrangement of keratin filaments in the 2 h after raising the calcium ion concentration. During this time they extended towards the cell periphery and filaments in adjacent cells became aligned (Fig. 1 *i*).

### Stability of Desmosomes

There are conflicting reports in the literature about the sensitivity of desmosomes to calcium depletion or EDTA treatment (1, 13, 16, 30, 31). We investigated this by exposing keratinocyte monolayers to normal medium (2.0 mM calcium) for 2–12 h and then treating them with 0.6 mM EDTA. When keratinocytes that had been in normal medium for 2 h were incubated in EDTA, the cells separated from one another, rounded up, and came off the culture dish as a single cell suspension (Fig. 2 *a*). However, if the cells had been in normal medium for 3 h or more, they rounded up in EDTA, but remained attached to one another at regions that stained positively for desmosomal antigens; eventually the cells detached from the culture substratum as an intact sheet (Fig. 2 *b*). This suggests that some change in desmosomes occurs several hours after they have formed, which makes them resistant to breakdown by EDTA.

We also examined the effect on desmosomal antigens of returning keratinocytes to low-calcium medium. Keratinocytes were incubated in normal medium for 2–8 h and then transferred to low-calcium medium for 24 h. At the end of this time, many cells no longer had a punctate pattern of desmosomal components. If keratinocytes were induced to stratify by 24-h incubation in normal medium, desmosomal staining became reduced within 1 d of returning the cells to low-calcium medium (Fig. 2 *c*); and after 2 d, the suprabasal cells often began to detach. It seems, therefore, that calcium may be required for the maintenance of desmosomes, as well as for their formation.

### Stratification and Cell Sorting

Within 9 h of raising the level of calcium ions, some keratinocytes began to move out of the basal layer. Regions of overlap between these cells and cells that remained on the culture substratum were characterized by a punctate pattern of desmosomes (Fig. 3 *a*). After 24 h, stratification was complete and there were extensive, often linear, arrays of desmosomes connecting stratified cells with the underlying basal cells (Fig. 3 *b*). Surface staining of suprabasal cells with anti-100 and anti-115 was weaker than at 2 h and was confined mainly to the cell margins.

Movement out of the basal layer does not occur at random; instead, there is selective migration of terminally differentiating cells, which can be identified because they synthesize involucrin, a precursor of the cross-linked envelope (34, 35). Desmosomes have often been implicated in cell sorting, because cells with larger numbers of desmosomes are more cohesive than cells with fewer desmosomes (20, 23, 36). We were therefore interested to discover whether there was any difference in desmosomes formed by involucrin-negative and -positive keratinocytes before stratification.

Keratinocyte monolayers were exposed to normal medium for 2 h, to induce desmosome formation, and then stained with anti-205/230 and antiserum to involucrin. We found desmosomal staining between adjacent involucrin-positive cells, between adjacent involucrin-negative cells, and between involucrin-positive and -negative cells. However, there was no evidence of sorting-out in the plane of the monolayer (10, 19, 20, 32). All involucrin-positive cells showed increased cytoplasmic staining with anti-205/230. Often, involucrin-positive cells had begun to round up (35) and desmosomal staining at regions of contact was lacking (Fig. 4).

## DISCUSSION

Addition of calcium to keratinocyte monolayers triggers the assembly of desmosomes from their individual components

and a characteristic rearrangement of keratin filaments (13). Thus, a population of individual cells become associated into an integrated tissue. We investigated these events, using antisera that recognize the different desmosomal proteins.

We found that all the desmosomal proteins became localized at the cell periphery 15 min after calcium was added and continued to accumulate there for at least 2 h. This was accompanied by a progressive rearrangement of keratin filaments: they were no longer concentrated around the nucleus but extended to the cell margins, and keratin filaments in adjacent cells became aligned.

These results can be compared with ultrastructural data already published on the time required for desmosome assembly under different conditions. Hennings and Holbrook (13) reported that desmosomal plaques appear within 5 min of adding calcium to mouse keratinocyte monolayers. This is a little faster than in our system, but the discrepancy can probably be explained by differences in the culture conditions and species of cell. When cervical carcinoma cells are allowed to recover from trypsinisation before aggregation, complete desmosomes are formed within 90 min (7). However, desmosome formation takes several hours if cells are brought into contact immediately after treatment with trypsin (6, 21).

Rearrangement of desmosomal antigens also occurs in Madin-Darby bovine kidney cells (6). Here, over a period of several days, with the development of epithelial polarity, they

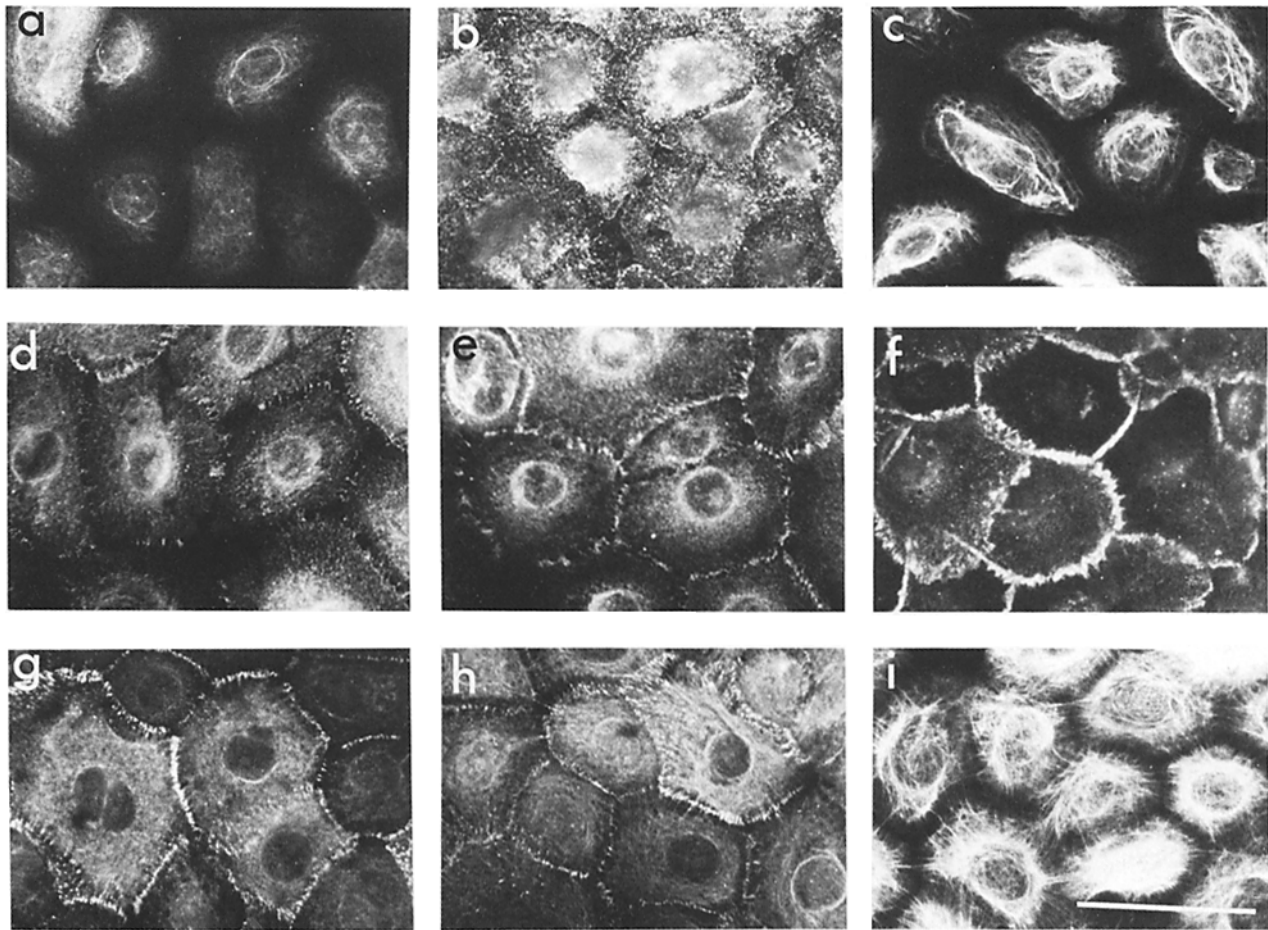
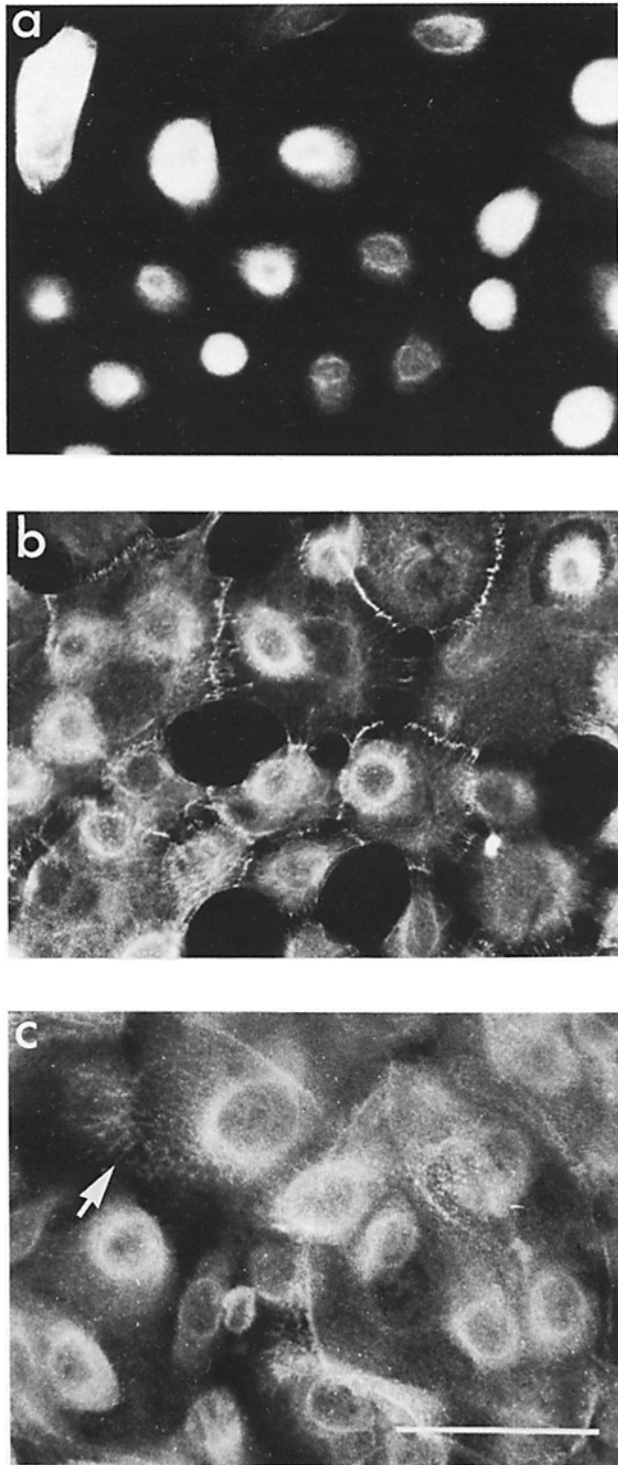


FIGURE 1 Calcium-induced rearrangement of desmosomal antigens and keratin filaments. (a-c) Keratinocytes in low-calcium medium: a, anti-205/230; b, anti-100, live staining for 30 min at 37°C before fixation; c, LP34. (d) 15 min after adding calcium, anti-205/230. (e) 30 min after adding calcium, anti-82/86. (f) 90 min after adding calcium, anti-100. (g-i) 2 h after adding calcium: g, anti-205/230; h, anti-150; i, LP34. Bar, 50  $\mu$ m.  $\times$  490.

gradually become confined to the lateral and basal cell surfaces. Thus the upper surfaces of the cells become nonadhesive, as might be expected of cells whose role *in vivo* was to form tubules. In contrast, rearrangement in keratinocytes, though extremely rapid initially, did not proceed to completion. Up to the time of onset of stratification (9 h after

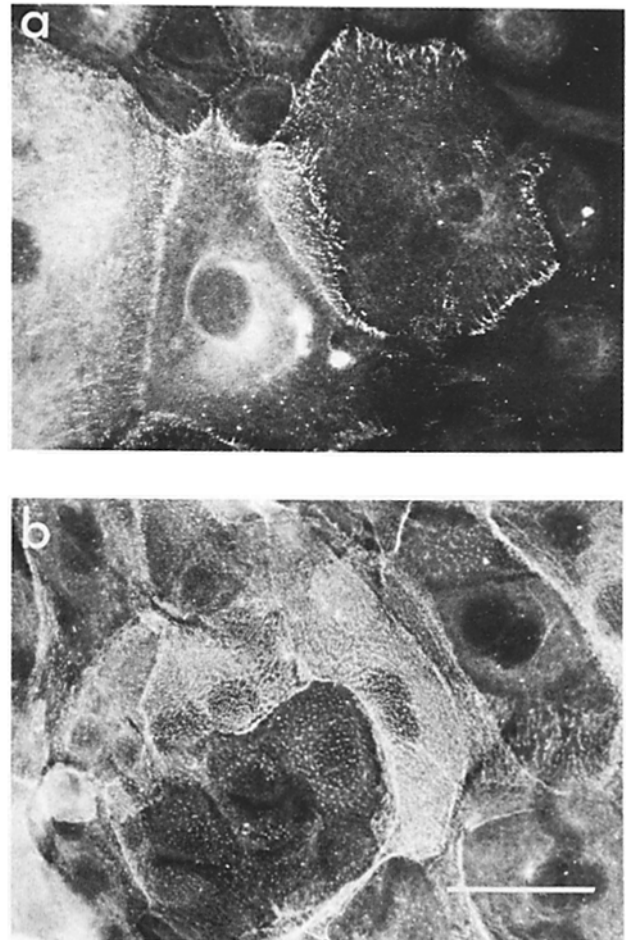


**FIGURE 2** Stability of desmosome formation. (a and b) Effect of EDTA on keratinocyte monolayers incubated in normal medium for (a) 2 h and (b) 3 h. (c) Keratinocytes incubated in normal medium for 24 h, and then in low-calcium medium for 24 h. Arrow indicates remaining desmosomal staining. (a-c) Anti-205/230. Bar, 50  $\mu$ m.  $\times$  530.

increasing the calcium concentration) and beyond, there were still detectable amounts of free 100,000- and 115,000-mol-wt antigens on the cell surface. This may represent a functional requirement for stratification and a fundamental difference in organization between keratinocytes and Madin-Darby bovine kidney cells.

Our experiments provide information about the location of individual desmosomal components before junction formation. The cytoplasmic plaque constituents (205,000 and 230,000 mol wt) and the 82,000- and 86,000-mol-wt proteins were found throughout the cytoplasm, and the level of cytoplasmic staining decreased after addition of calcium. The presence of 100,000- and 115,000-mol-wt glycoproteins on the cell surface is consistent with their proposed role in cell adhesion (6).

Antibodies raised against bovine desmosomal proteins cross-react with the desmosomes in many tissues of other vertebrates, demonstrating that the proteins are highly conserved (4, 5, 8). However, a difference in sensitivity to EDTA between desmosomes of simple columnar epithelia and those of stratified squamous and many glandular epithelia has been reported (1). It is therefore possible that desmosomes of the latter category, of which keratinocyte desmosomes are an example, undergo an additional stabilizing event after formation. Our experiments support this conclusion: rapid breakdown of desmosomes could be induced by EDTA in



**FIGURE 3** Calcium-induced stratification. Addition of normal medium to keratinocyte monolayers for (a) 9 h and (b) 24 h. (a and b) Anti-205/230. Bar, 50  $\mu$ m.  $\times$  390.

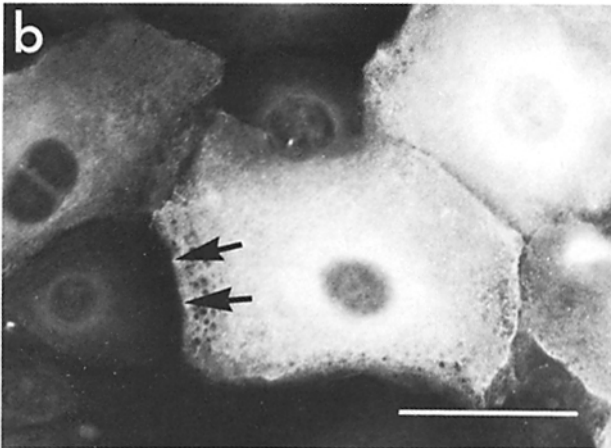
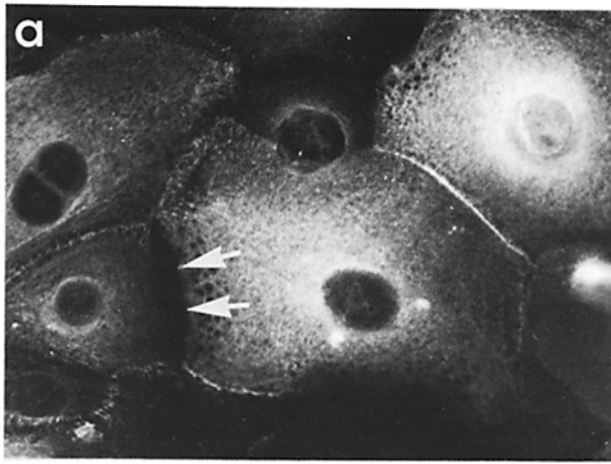


FIGURE 4 Double label immunofluorescence with (a) anti-205/230 and (b) antiserum to involucrin. Arrows indicate cell margin lacking desmosomal staining. Keratinocytes exposed to normal medium for 2 h. Bar, 50  $\mu\text{m}$ .  $\times 480$ .

cultured keratinocytes 2 h after raising the calcium ion concentration, but by 3 h the desmosomes had become resistant to EDTA.

During calcium-induced stratification of keratinocytes, cells that are linked laterally by desmosomes rearrange themselves so that terminally differentiating, involucrin-positive cells come to lie above the basal cell layer (34, 35). This could be achieved in two ways: either by rearrangement of existing desmosomes or breakdown of desmosomes on lateral cell surfaces with reformation at the surfaces of contact between basal and suprabasal cells. Our observation that involucrin-positive cells in the monolayer often had fewer desmosomes than involucrin-negative cells and had brighter cytoplasmic staining with anti-205/230 may indicate that desmosome breakdown is the more important mechanism. Although desmosomes are probably essential for stratification, it is not clear whether they confer selectivity of entry into the suprabasal layers. Changes in nonjunctional membrane glycoproteins during terminal differentiation may alter the cells' adhesive properties (33), and the decreased affinity of involucrin-positive cells for substratum attachment may be more important for selective migration than their altered cohesiveness (34).

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