

Organization of Desmosomal Plaque Proteins in Cells Growing at Low Calcium Concentrations

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Abstract. Desmosomes are not formed in epithelial cell cultures growing in media with low (≤ 0.1 mM) concentrations of Ca^{2+} (LCM) but appear rapidly upon shift to media of normal calcium concentrations (NCM). Previous authors using immunolocalization of desmoplakin, a marker protein for the desmosomal plaque, in LCM-grown cells have interpreted positively stained, dense, cytoplasmic aggregates on intermediate filaments (IF) bundles as preformed plaque units which upon NCM shift would move to the plasma membrane and contribute to desmosome formation. Studying various cell cultures, including primary mouse keratinocytes and human A-431 cells, we show that most, probably all, desmoplakin-positive aggregates in LCM-grown cells are associated with membranous structures, mostly vesicles, and also contain other desmosomal markers, including desmoglein, a transmembrane glycoprotein. We interpret such vesicles as residual desmosome-derived domains endocytosed upon cell dissociation. Only keratinocytes grown

for long times (2–4 wk) in LCM are practically free from such vesicles. In addition, we demonstrate that certain cells such as A-431 cells, when passaged in LCM and in the absence of stable junctions, are able to continually assemble “half-desmosomes” on the plasma membrane which in turn can be endocytosed as plaque-bearing vesicles. We also show that in LCM the synthesis of several desmosomal proteins (desmoplakins I and II, plakoglobin, desmoglein, “band 6 protein”) continues and that most of the plaque protein, desmoplakin, is diffusely spread over the cytoplasm, apparently in a soluble monodisperse form of $\sim 9\text{S}$. From our results we propose that the plaque proteins occur in small, discrete, diffusible entities in the cytoplasm, in concentrations that are relatively high in LCM and low in NCM, from which they assemble directly, i.e., without intermediate precursor aggregates on IFs in the cytoplasm, on certain plasma membrane domains in a Ca^{2+} dependent process.

INTERACTION and communication between vertebrate cells, notably the formation and maintenance of tissues, is largely dependent on semi-stable linkage structures, the intercellular junctions. Among the major categories of junctions (gap junctions, adhering junctions, tight junctions) the junctions of the *adhaerens* category are of particular interest in our understanding of cell and tissue morphology, as they are involved not only in cell–cell connections but also in the cell type-specific spatial organization of the filamentous cytoskeleton. Cytoplasmic plaques of adhering junctions of the desmosomal kind provide anchorage sites for the attachment of intermediate filaments (IFs) whereas the non-desmosomal junctions (*zonulae* and *fasciae adhaerentes*, *puncta adhaerentia*) are usually associated with actin micro-

filaments (22, 61). Primarily due to the availability of procedures for isolating sufficient quantities of desmosomes from certain stratified epithelia (13, 17, 19, 25, 59), special progress has been made in the identification of major desmosomal constituent proteins, including obligatory ones such as the plaque proteins, desmoplakin I ($M_r \sim 250,000$; 18, 46), and plakoglobin ($M_r \sim 83,000$; 10, 26; this protein also occurs in plaques of nondesmosomal adhering junctions; 11), and the transmembrane glycoprotein, desmoglein ($M_r \sim 165,000$; “band 3” polypeptide of isolated desmosomes from bovine muzzle epidermis; 6, 17, 25, 55, 56, 62), and “accessory” components not found in desmosomes of all tissues, including plaque proteins such as desmoplakin II and the basic “band 6 polypeptide” (cf. 9, 10, 35) as well as glycoproteins (6, 7, 8, 17, 24, 25, 43, 52, 53). Further minor and/or cell type-specific desmosome-associated components have been described (e.g., 62, 63). This compositional complexity suggests that the formation of the structure called desmosome requires the coordination of the synthesis and assembly of various transmembrane proteins and proteins exclusive to the

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1. *Abbreviations used in this paper:* IF, intermediate filaments; LCM, low calcium concentrations; NCM, normal calcium concentrations.

cytoplasmic compartment as well as of cell-type specific constituents.

Earlier electron microscopic studies have described the advent of desmosome-like structures during early embryogenesis (31, 40) and in cell cultures (e.g., 12; for reviews see references 9 and 39). It has also been observed, in certain tissues and in cell cultures depleted of calcium or treated with trypsin, that asymmetric plasma membrane domains equivalent to "half-desmosomes" are formed and rapidly internalized in the form of special plaque-bearing vesicles, usually with IF bundles still attached, resulting in intracellular accumulations of desmosome-derived vesicles (17, 36, 37, 42, 49; for reviews see also 9, 39). These and other studies have indicated that desmosomes, like other intercellular junctions, are dependent on calcium, at least in cell culture systems. Specifically, various epithelial cell cultures, when grown in media with low calcium concentrations (LCM; ≤ 0.1 mM), do not form desmosomes but do so within a relatively short period of time upon shift to normal calcium concentrations (NCM; cf. 27-29, 32).

Using mouse keratinocyte cultures grown in LCM and immunolocalization techniques Jones and Goldman (32) have described reactions of desmoplakin antibodies with small spheroidal structures ("dots") abundantly present in the cytoplasm in association with IF bundles, and concluded that desmoplakin exists, in LCM-grown cells, in preformed aggregate structures on IF bundles which then, upon shift to normal calcium levels, move "as a package" to the cell surface, thus forming the typical desmosome-IF bundle architecture. As this interpretation was at variance with several observations made by us and other authors (e.g. 3, 41) we studied the state of desmosomal proteins in various cell cultures growing in LCM, including primary cultures of mouse epidermal keratinocytes. We report here that for several days, desmoplakin, together with other desmosomal proteins, can be identified in plaques attached to intracytoplasmic vesicles and in association with IFs, probably resulting from endocytotic uptake of previously existing desmosomal domains, but that given sufficient time of recovery, all desmoplakin and plakoglobin is diffusely spread over the cytoplasm in no obvious association with IFs. In addition, we describe that "half-desmosomes" with typical plaques and IFs attached can be formed in LCM cultures of certain cell lines. We also show that in such LCM cultures a considerable portion of the desmosomal plaque proteins occurs in distinct soluble forms which are rapidly recruited for the de novo formation of desmosomes, apparently in loco, i.e., at the plasma membrane, upon shift to NCM.

Materials and Methods

Cell Culture

Primary cultures of epidermal keratinocytes were prepared from newborn NMRI-mouse skin by the trypsin-flotation procedure (65). The cells were plated in plastic dishes (Falcon, Oxnard, CA) in "normal Ca^{2+} medium" (NCM; DME containing 1.8 mM Ca^{2+} , supplemented with 10% FCS) at a density of $\sim 1 \times 10^6$ cells per dish (5 cm diameter). After 6 h, the medium was removed, and cultures were washed once with Ca^{2+} -, Mg^{2+} -free PBS to remove unattached cells. The cells were fed with "low Ca^{2+} medium" (LCM; 3 vol DME and 1 vol HamF 12, without Ca^{2+} ; from Biochrom, Berlin-West), supplemented with 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin, and 10% Chelex-treated FCS (Chelex treatment was performed according to reference 5, with Chelex 100-resin; Bio-Rad Laborato-

ries, Richmond, CA), adjusted to 0.07 mM CaCl_2 at this time-point and thrice weekly thereafter. LCM cultures could be maintained at near confluency for several months. Cell cultures were used for experiments at different times after plating.

Human A-431 cells, a permanent line derived from an epidermoid carcinoma of the vulva, and cells of Madin-Darby bovine kidney (MDBK) and Madin-Darby canine kidney (MDCK) lines were grown in normal medium (see 16, 18, 45) or LCM, with serial passages every third day. Routinely, cells on the third day after passage were used for experiments.

The Ca^{2+} concentrations in the specific culture media were determined by atomic absorption spectrophotometry and adjusted to the desired level by addition of the appropriate volume from a sterile 10 mM CaCl_2 solution.

Antibodies

Four previously described mouse monoclonal antibodies were routinely used: anti-desmoglein DG3.10 (55, 56), anti-desmoplakin I-2.17 (10), anti-plakoglobin PG5.1 (11) and anti-cytokeratin lu-5 (20). In some experiments we used a mixture of murine monoclonal desmoplakin antibodies DPI and II-2.15, 2.17 and 2.19 (10). Affinity-purified guinea pig antibodies (IgG fraction) against the known major desmosomal proteins were used for comparison (10, 11, 17, 18, 35, 46, 55, 56).

Immunofluorescence Microscopy

Cells grown in NCM or LCM on uncoated (mouse keratinocytes) or on poly-L-lysine-coated (A-431 cells in LCM) coverslips were fixed for 9 min in methanol (-20°C), followed by a brief immersion (1 min) in acetone (-20°C), and air-dried. Alternatively, cells gently lysed by incubation with 0.01% saponin in PBS for 10 min were washed three times, 5 min each, with PBS and incubated directly with the antibodies. Primary antibodies were applied for 30 min, followed by three 5-min washes in PBS, 30 min of incubation with Texas Red-coupled goat anti-mouse or anti-guinea pig Igs (Medac, Hamburg, FRG) diluted 1:150, and three 5-min washes in PBS. The coverslips were dipped briefly in water, then in ethanol, and were mounted in Mowiol (Hoechst, Frankfurt, FRG). For double-label immunofluorescence microscopy, primary and, subsequently, secondary antibodies were applied as a cocktail. Texas red-coupled goat anti-mouse Igs were used in cases of primary murine antibodies and FITC-coupled goat anti-guinea pig Igs for primary antibodies of guinea pig origin. To prevent photobleaching of FITC, *p*-phenylenediamine (1 mg/ml) was added to the mounting medium. The cells were viewed, using epifluorescence illumination, with a Zeiss photomicroscope III (Carl Zeiss, Oberkochen, FRG).

Immunofluorescence microscopy of mouse keratinocytes grown for prolonged periods of time in LCM was performed with cells grown on culture dishes rather than on glass coverslip-attached cells. In that case, cells were fixed only with methanol (-20°C , 10 min) and air-dried (for details and further processing see reference 14).

Electron Microscopy

Cells grown on glass coverslips or on Falcon culture dish surfaces were shortly rinsed with PBS and fixed sequentially with solutions containing 2.5% glutaraldehyde and 1% OsO_4 , dehydrated using an ethanol series, embedded in Epon, and ultrathin-sectioned as described (15). Electron micrographs of ultrathin sections were taken at 80 kV using a Zeiss EM10 electron microscope (Carl Zeiss, Oberkochen, FRG).

For immunoelectron microscopy, the interior of cells grown on glass coverslips was made accessible by gentle treatment with PBS containing 0.01% saponin and 1 mM MgCl_2 for 10 min, followed by three washes in PBS for 5 min each, before and after incubation with primary and secondary antibodies. Incubation times were 30 min for the primary antibodies and 4 h for the secondary antibodies which were either anti-mouse or anti-guinea pig Igs conjugated to colloidal gold particles (5 or 10 nm diameter; Janssen, Beerse, Belgium), diluted 1:10 in PBS. For double-label localization 5 nm gold-conjugated anti-mouse Ig and 10 nm gold-conjugated anti-guinea pig Ig were simultaneously used. After incubation with antibodies, specimens were washed with PBS, postfixed with glutaraldehyde and OsO_4 , embedded in Epon and ultrathin-sectioned as described (11, 37).

Preparation of Cytoskeletons and Cell Fractionation Procedures

For the fractionation of cell components into pelletable ("insoluble") and

nonpelletable ("soluble") proteins the procedures used in our laboratory for the detection of small amounts of soluble forms of cytoskeletal proteins were applied with minor modifications (11, 33–35, 60). For all steps cold buffer solutions (~4°C) were used. Confluent cell cultures were rinsed in buffer of "near-physiological" ionic strength and pH (140 mM NaCl, 2.7 mM KCl, 5 mM EDTA, 6.5 mM NaH₂PO₄, 1.5 mM KH₂PO₄, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM ϵ -aminocaproic acid, pH 7.4). Then 1 ml of the same buffer containing 0.05% saponin was added per 5-cm dish monolayer culture to lyse the cells. After 10-min incubation, cells were gently scraped off with a rubber policeman and homogenized by 20 strokes with a Dounce glass homogenizer. Low speed pellets obtained after centrifugation of the homogenate for 10 min at 4,000 g were saved and supernatant fractions were centrifuged further for 2 h at 100,000 g. The resulting high speed pellet, the 100,000 g supernatant and the low speed pellet were processed for gel electrophoresis and immunoblotting.

Sucrose Gradient Centrifugation

Proteins of the 100,000 g supernatant fraction described above were concentrated by vacuum dialysis against near-physiological salt buffer to a final volume of 500 μ l (33, 34, 60). These samples were then loaded on top of ~11 ml of a 5–30% (wt/vol) continuous sucrose gradient and centrifuged in a Beckman SW40 rotor at 35,000 rpm for 18 h at 5°C. Fractions of 400 μ l were collected with an Isco gradient fractionator model 640 (Isco, Lincoln, NE), and the proteins were precipitated with 10% trichloroacetic acid (TCA), washed twice with acetone, air-dried, and analyzed for the presence of desmosomal proteins by SDS-PAGE and immunoblotting (for details see reference 14).

Immunoprecipitation of Desmosomal Proteins

The protocol used followed the principles described by Blose and Meltzer (2), with some modifications as described by Penn et al. (52, 53). Cells growing in 5-cm Falcon culture dishes were labeled for 3 h with 400 μ Ci [³⁵S]methionine (800 Ci/mmol Amersham International, Amersham, UK) in DME without methionine and Ca²⁺, supplemented with either 10% Chelex-treated FCS or 10% FCS, after a 30-min preincubation in the same medium. The Ca²⁺-concentration of the labeling medium was adjusted to either 0.07 mM (for LCM) or 1.8 mM (for NCM). After labeling, the cells were washed once each with medium and PBS, homogenized in 250 μ l of sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol) and boiled for 10 min. The homogenates were centrifuged for 10 min at 10,000 g. Aliquots of the supernatant were diluted to 0.1% SDS with dilution buffer (1% Nonidet NP-40, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA) and reacted with either 2 μ l of antiserum or 5 μ l ascites fluid (in the case of monoclonal antibodies) for 2 h at 4°C. 50 μ l (5 mg) of a suspension of protein A-Sepharose in "immunoprecipitation buffer" (0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 150 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl, pH 7.5) were added, and this was incubated for another 3 h. In the case of antibody DG3.10, which does not bind protein A, protein A-Sepharose was complexed with rabbit anti-mouse Ig (Conco, Wiesbaden, FRG) for 2 h before addition. Negative controls were performed using protein A-Sepharose alone or with other antibodies. Finally, immunoprecipitates obtained were washed three times with immunoprecipitation buffer and once with PBS, and the final pellets were boiled in SDS-PAGE sample buffer containing 5% β -mercaptoethanol.

Gel Electrophoresis and Immunoblotting

SDS-PAGE on 7.5% or 10% polyacrylamide slab gels and two-dimensional gel electrophoresis were as described (46). Labeled polypeptides of immunoprecipitates were visualized by autoradiography of the dried gels after treatment with ³Enhance (NEN, Dreieich, FRG) for 1 h. For immunoblotting, gel electrophoretically separated polypeptides were transferred to nitrocellulose and reacted with antibodies as described (10). Affinity purified, ¹²⁵I-labeled goat antibodies to mouse IgG and IgM (10⁵ cpm/ml) or ¹²⁵I-labeled protein A (10⁵ cpm/ml; Amersham) were used as secondary Ig-binding reagent. Reactivity was visualized by autoradiography.

For immunoblot analysis of proteins of pellets and supernatant fractions described above, the separated polypeptides were transferred to nitrocellulose, stained with Ponceau S (Sigma, Taufkirchen, FRG), and the nitrocellulose paper sheets were cut horizontally into three strips containing the respective molecular weight regions of desmoplakin, desmoglein or plakoglobin. These strips were incubated separately with primary antibodies directed against the respective antigen, to analyze for the presence of

these three antigens in the same experiment, followed by IgG detection and autoradiography.

Results

Light and Electron Microscopy

Cells Showing Dispersed Desmosomal Proteins in LCM. Primary mouse keratinocytes grown in LCM do not possess desmosomes, as demonstrated by electron (28, 29) and immunofluorescence microscopy (Fig. 1 a; cf. 3, 32, 42, 43, 64). To study the appearance of desmosomal plaque proteins during desmosome formation we processed mouse keratinocyte cultures, taken at different time points after plating in LCM, for immunofluorescence and electron microscopy. We found, by immunofluorescence microscopy using anti-desmoplakin and anti-plakoglobin, that cells grown in LCM for short (e.g. 2–3 d) and long (2–4 wk) periods of time displayed very different distributions of the plaque proteins. After 4 wk of culture in LCM, immunofluorescence staining obtained with both antibodies to desmoplakin (Fig. 1 a) and to plakoglobin (not shown) was of moderate intensity and diffusely spread throughout the cytoplasm. After shift to NCM, the immunostaining was still rather homogeneously dispersed over the cytoplasm at 5 min, as shown for both desmoplakin (Fig. 1 b) and plakoglobin (Fig. 1 c), but within the subsequent 15–30 min, punctate staining arrays along cell boundaries as typical for desmosomes (17, 18) were seen with antibodies to both desmoplakin (Fig. 1 d) and plakoglobin (Fig. 1 e). In addition, however, some diffuse cytoplasmic fluorescence was still noticed. During this time we did not observe an intermediate state with accumulations of cytoplasmic dots. The clearance of most of this diffuse fluorescence from the cell interior progressed with increasing time of culture in NCM, and was inconspicuously low at 21 h after shift to NCM (Fig. 1 f) when practically all immunostaining of desmoplakin appeared at the cell periphery in desmosomal patterns (Fig. 1 f). This indicates that in undisturbed NCM cell cultures the steady state proportion of soluble desmoplakin is rather low, compared with LCM concentrations. With plakoglobin antibodies, the residual level of diffuse cytoplasmic immunofluorescence was somewhat higher, corresponding to observations in tissues and the known occurrence of sizable soluble pools of this protein (cf. 11, 33).

By contrast, cells grown only for 48 h in LCM showed a different picture in that both plaque proteins, desmoplakin (Fig. 2 a) and plakoglobin (not shown), were concentrated in brightly fluorescent cytoplasmic dots which tended to be enriched in the juxtannuclear region. By double label immunofluorescence microscopy, many of these spots appeared to be located at cytokeratin IF bundles (*arrowheads* in Fig. 2, a and b). In similar double-label experiments, sites of colocalization of desmoplakin and plakoglobin were demonstrated although, as expected (11), sites positive only for plakoglobin were also encountered (data not shown; for details see reference 14). This punctate distribution was very similar to that reported by previous authors for the same (27, 32) and other (e.g., 41, 64) cell cultures grown in LCM.

However, similarly abundant cytoplasmic dots of staining with antibodies to desmosomal plaque proteins, often showing perinuclear accumulations, had also been noted previ-

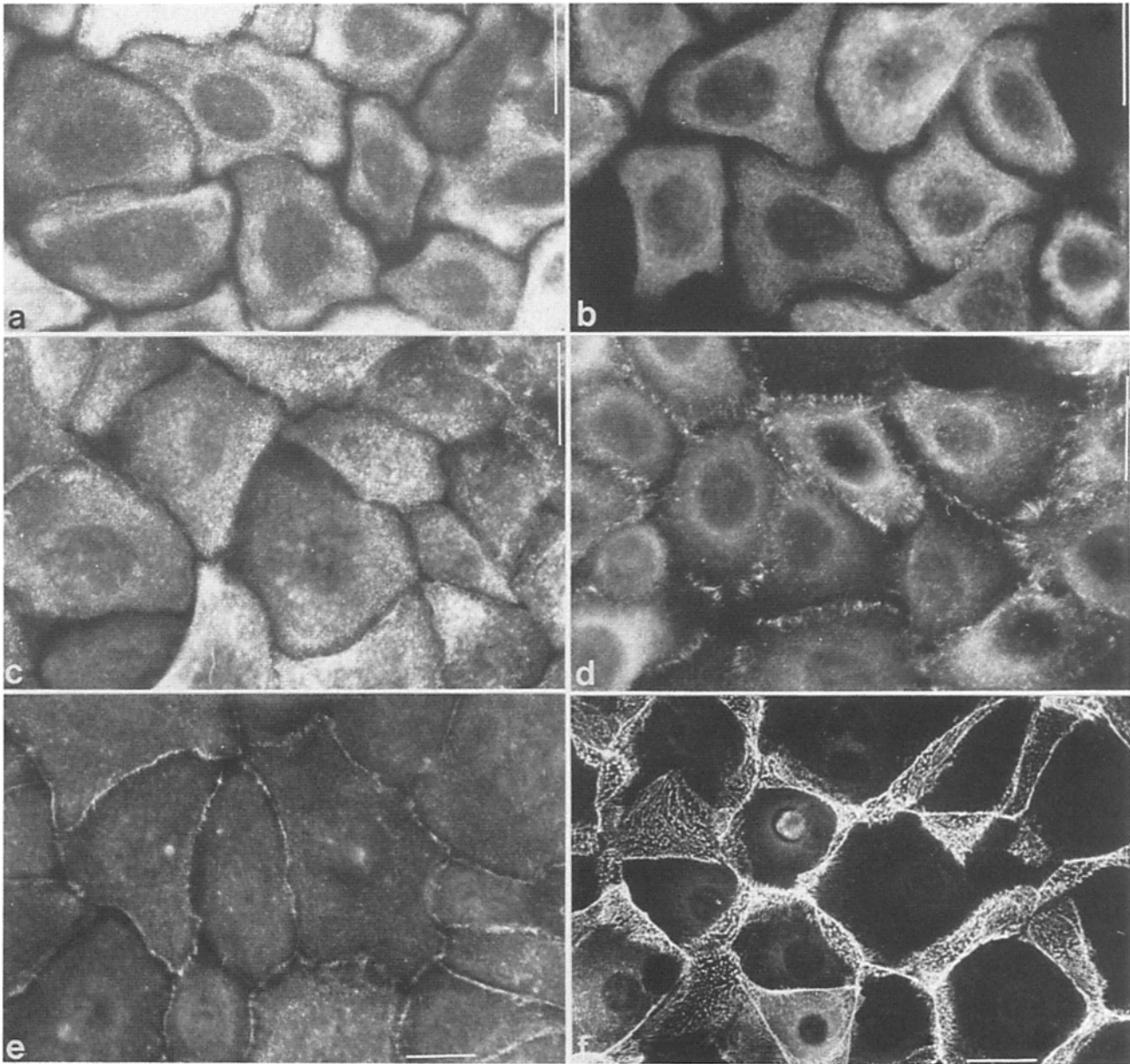


Figure 1. Immunofluorescence microscopy of primary mouse keratinocytes kept for 4 wk in LCM, after reaction with anti-desmoplakin (*a*, *b*, *d*, and *f*) and anti-plakoglobin (*c* and *e*). LCM-grown cells (*a*) and cells at 5 min (*b* and *c*), 30 min (*d* and *e*) and 21 h (*f*) after shift to NCM are shown. Note the diffuse cytoplasmic fluorescence for desmoplakin in unshifted cells (*a*). 30 min after a shift to NCM, the desmosomal staining at cell boundaries is prominent for both desmoplakin (*d*) and plakoglobin (*e*) but some diffuse cytoplasmic fluorescence is still notable (cell boundary staining in *e* shows both desmosomes and other adhering junctions; cf. reference 13). With prolonged exposure to NCM diffuse cytoplasmic fluorescence for desmoplakin is further reduced (*f*). Bars, 25 μ m.

ously in our laboratory in many other cell lines passaged in NCM, for several days after normal serial dissociation and plating (e.g., 17, 18), where these dots had been shown to represent endocytosed, desmosome-derived vesicles, similar to those described in certain cell cultures upon depletion of extracellular calcium (36, 37, 42). Therefore, we examined the possibility that the cytoplasmic punctate arrays observed for up to several days upon shift to LCM might represent, compositionally, equivalents of entire desmosomes and not merely aggregates of precursor forms of cytoplasmic plaque

proteins. To this end, we applied double-label immunofluorescence microscopy combining antibodies to a cytoplasmic plaque protein, desmoplakin, with those specific for the transmembrane glycoprotein marker, desmoglein. Typical results of such experiments are shown in Fig. 3, *a-c*. In mouse keratinocyte cultures grown for 2 d in LCM, desmoglein also appeared in punctate arrays dispersed over the cytoplasm (Fig. 3 *a*), and double-label immunofluorescence microscopy showed many dots positive for desmoglein (Fig. 3 *b*) to be also positive, in the same focal plane, for desmoplakin (Fig. 3 *c*) and for plakoglobin (not shown; cf. 14). Careful focusing series further revealed that, in a number of

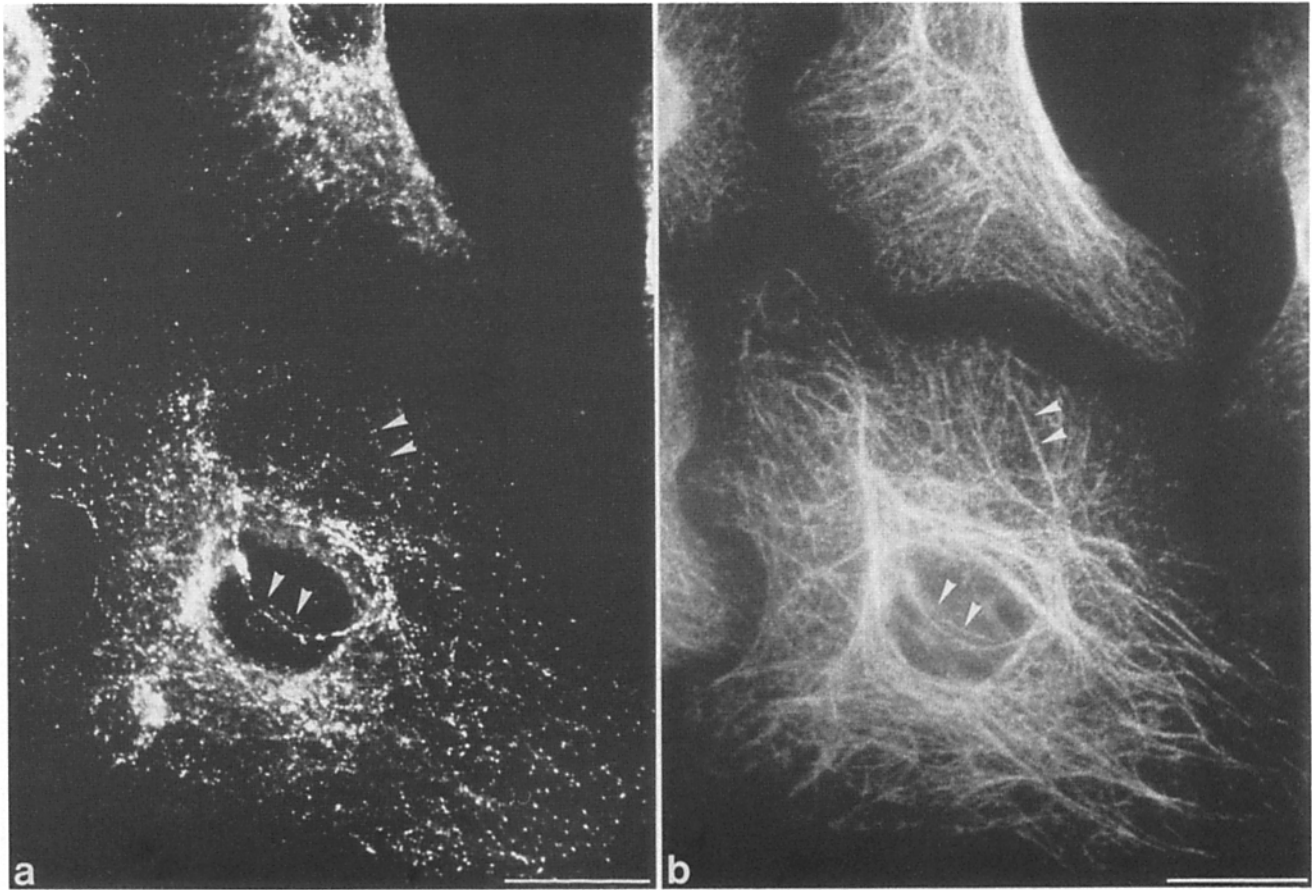


Figure 2. Double-label immunofluorescence of primary mouse keratinocytes, two days after plating in LCM, as seen with guinea pig antibodies to desmoplakin (*a*) and mouse antibody to cytokeratin (lu-5; *b*). Note the frequency of desmoplakin-positive "dots" deep in the cytoplasm, often in association with cytokeratin IFs (e.g., at arrowheads), and in juxtannuclear accumulations. High contrast print (*a*) is shown to emphasize the dots on the account of reduced diffuse desmoplakin fluorescence which is seen in the original photograph. Bars, 25 μ m.

sites, the positivity for the plaque protein was slightly displaced from the reactive site of the membrane marker, indicating that the distribution of the two antigens often is very similar but does not precisely overlap. The frequent colocalization of transmembrane and plaque-specific proteins suggested that most, perhaps all, of the cytoplasmic dots represent complex structures comprising a membrane and a plaque attached to cytokeratin IF bundles.

Here we have presented the results obtained with murine keratinocytes in some detail, as this was the cell system in which the original observations of Jones and Goldman (32) had been made. However, results with human, bovine (MDBK) and canine (MDCK) epithelial cell lines were essentially identical (examples are seen in references 14 and 42).

In ultrathin sections through the cytoplasm of primary mouse keratinocytes grown for 2 d in LCM, we found numerous vesicles of various sizes, with attached "caps" of electron-dense cytoplasmic plaques, which often appeared to be enriched in the vicinity of the nuclear envelope (Fig. 4 *a*). Massive fleeces of bundles ("tonofibrils"; T in Fig. 4, *a-d*) were attached to the plaques of these vesicles (some are

shown in greater detail in Fig. 4, *b-d*). The sizes and the thicknesses of these plaques were variable, and individual vesicles with two distinct plaque caps were also occasionally seen (e.g., Fig. 4 *d*). Similar observations were made with MDBK, MDCK, and BMGE cells (not shown; for cells grown in NCM, see references 17 and 18).

To correlate the dots observed by immunofluorescence microscopy (Figs. 2 *a* and 3, *a-c*) with the vesicles seen by electron microscopy, we lysed the cells with saponin in the same way as used for the light microscopic immunolocalization (see Fig. 3 *a*), followed by immunoelectron microscopy using anti-desmoplakin (Fig. 4, *e-f*) or anti-desmoglein (Fig. 4 *g*). Immunogold label could be seen in association with clusters of densely stained material most of which could be positively identified as caps of cytoplasmic plaques associated with vesicles (Fig. 4, *e-g*), either directly in cross-sections or in grazing or serial sections.

Cells Showing Assembly of Desmosomal Proteins in LCM. To our surprise we noted that in certain cell lines, when grown in LCM (0.04 mM Ca^{2+}), desmosomal proteins were not completely dispersed but that considerable amounts of desmoplakin, plakoglobin, and desmoglein ap-

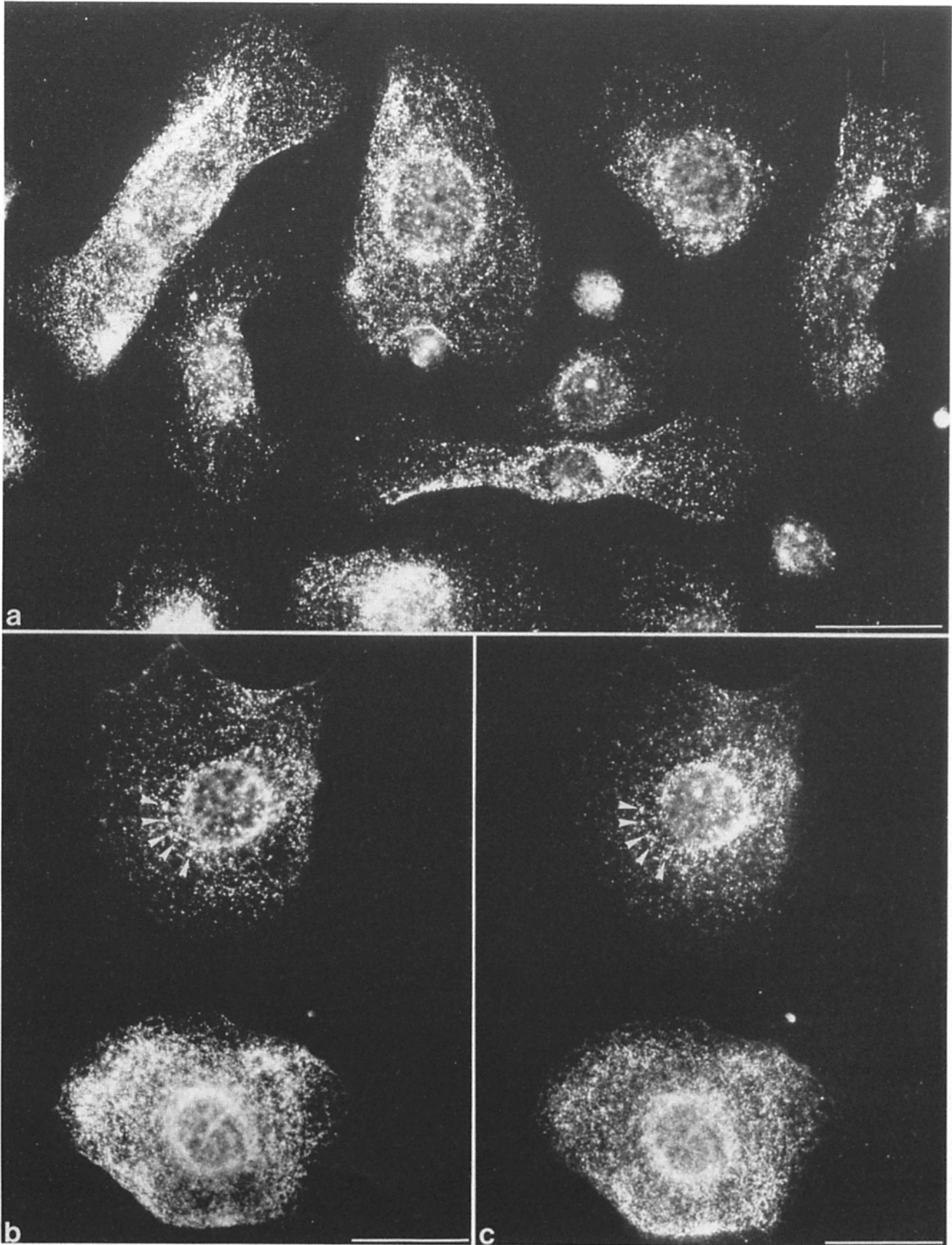


Figure 3. Immunofluorescence microscopic comparison of the localization of desmoglein (*a* and *b*) and desmoplakin (*c*) in primary mouse keratinocytes 2 d after plating in LCM. Cells were gently lysed with 0.01% saponin in PBS (*a*) or fixed with methanol and acetone (*b* and *c*) and reacted with murine anti-desmoglein (*a*; DG3.20) alone or, in a double-label experiment (*b* and *c*), in combination with guinea pig antibodies to desmoplakin (*c*). Note the numerous desmoglein-positive dots (*a* and *b*), indicative of the presence of desmosome-derived vesicles and the frequent localization of desmoglein (*b*) and desmoplakin (*c*) in the same dots, depending on the focal plane (some are denoted by arrowheads). Bars, 25 μ m.

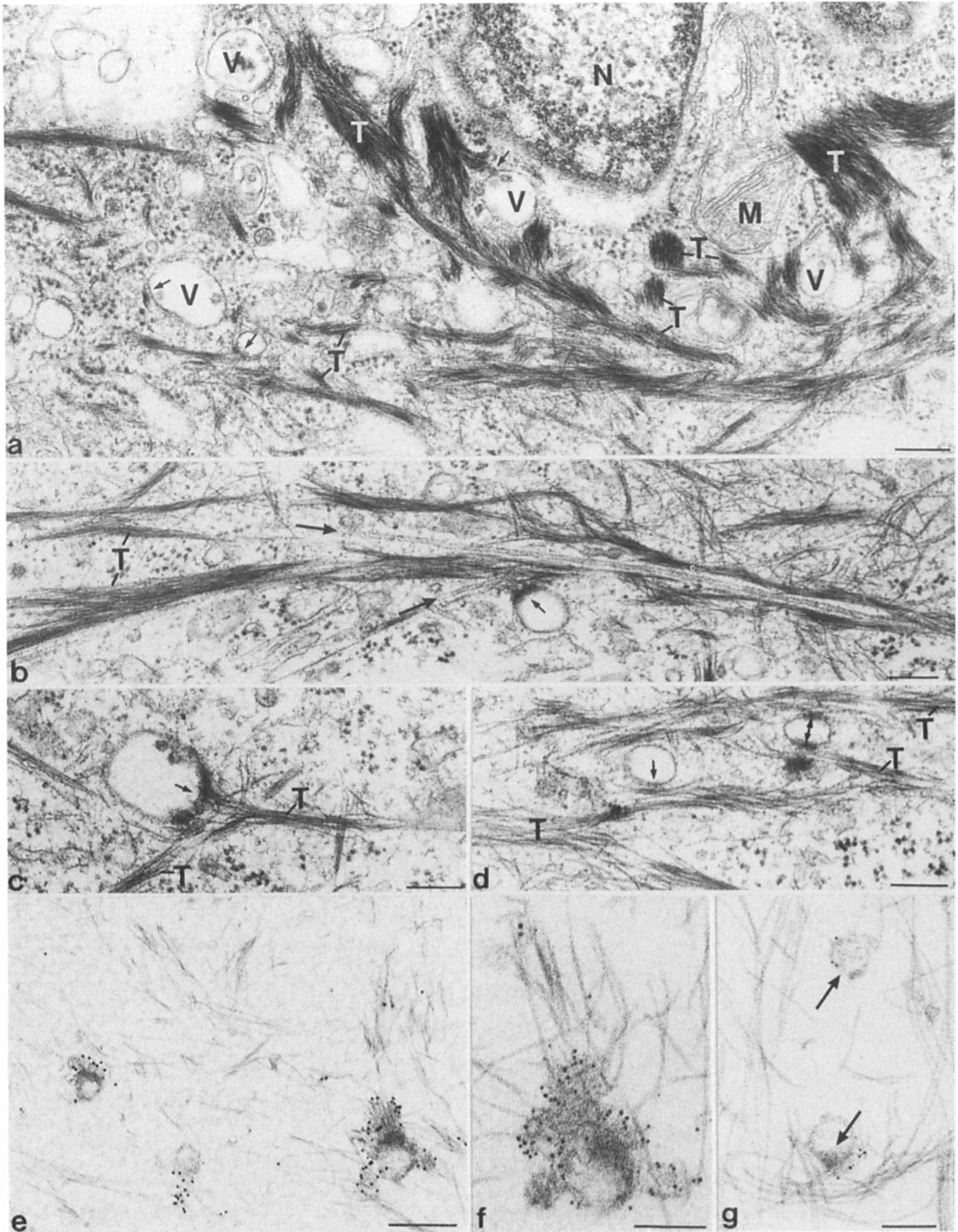
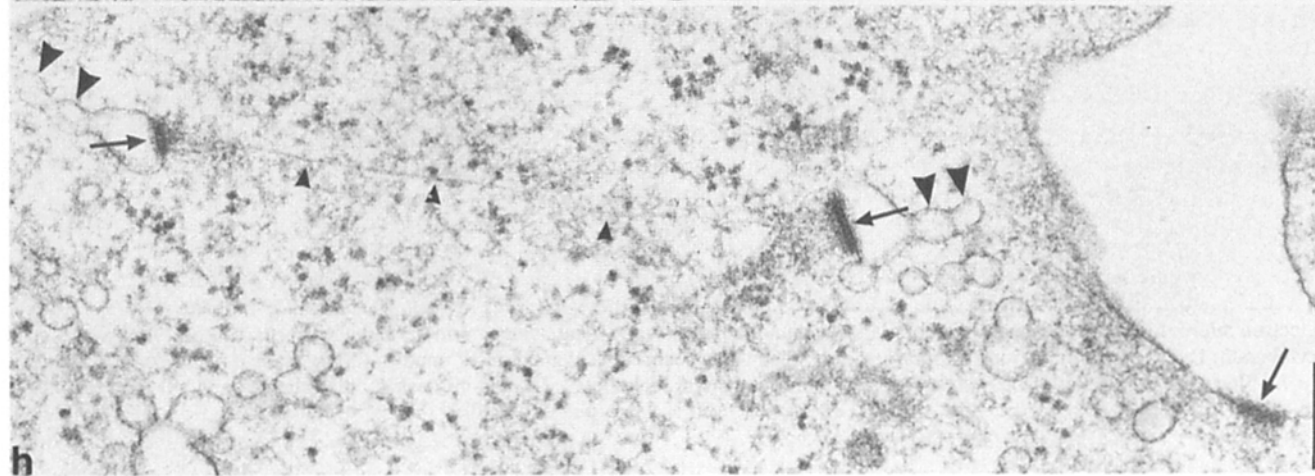
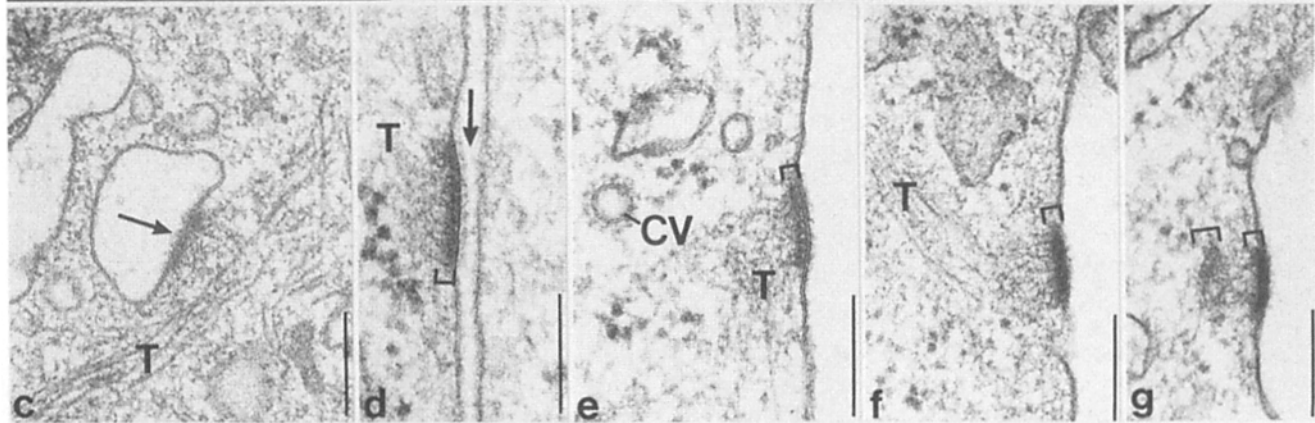
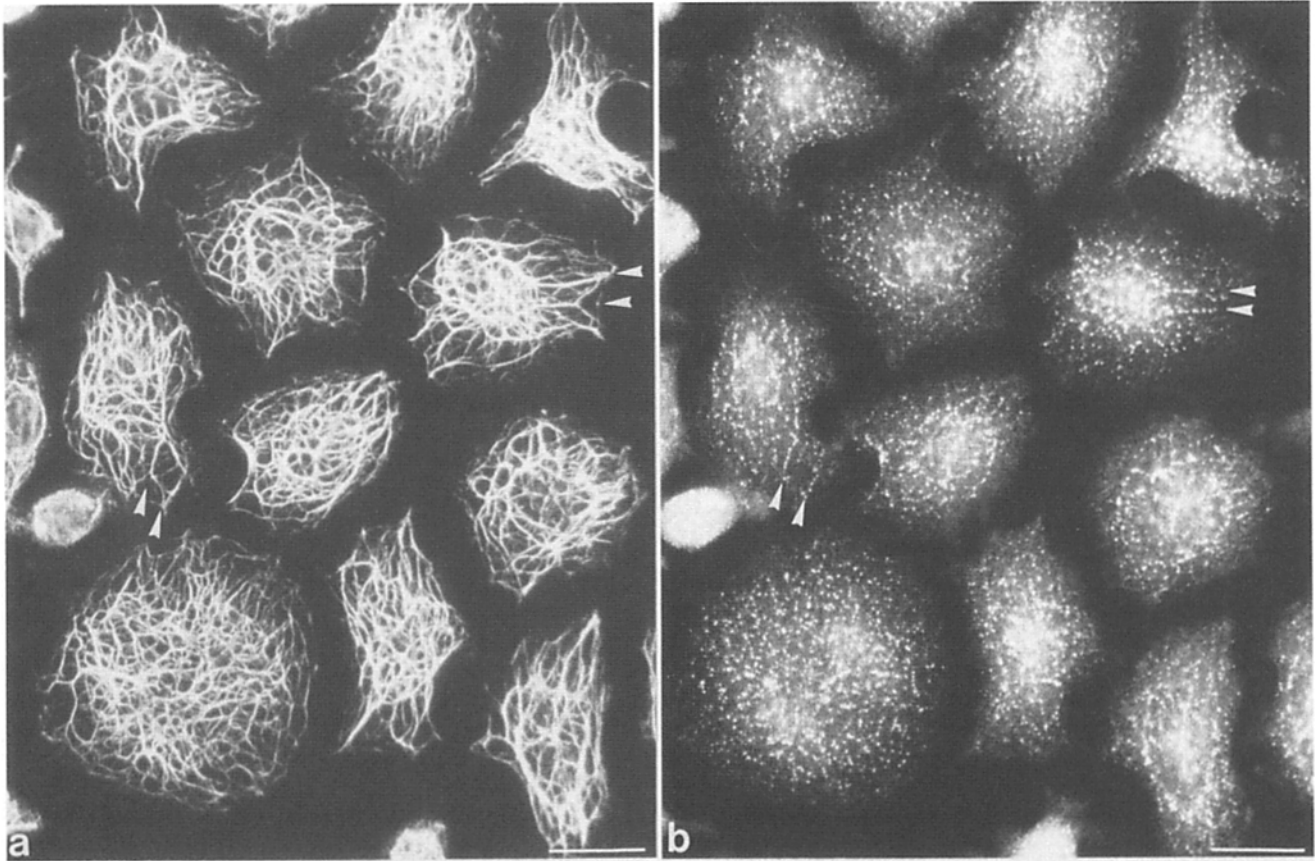


Figure 4. Electron (a-d) and immunoelectron (e-g) microscopy of ultrathin sections through primary mouse keratinocytes, 2 d after plating in LCM. Cells in (a-d), conventionally fixed, show the ultrastructure of IF-attached vesicles in the cytoplasm ([a] survey of perinuclear region; [N] nucleus; [V] vesicles with tonofibrils, [T]; [M] mitochondrion). In certain situations (some are denoted by arrows) dense plaques are resolved. (b-d) Details of vesicles and their attached plaques (thin arrows) and IF bundles (tonofibrils, T). The large arrow in (b) points to a microtubule parallel to an IF bundle. (e-g) Immunogold (5 nm) label in cells gently lysed with 0.01% saponin in PBS, reacted with guinea pig antibodies to desmoplakin (e and f) and monoclonal antibody DG3.10 to desmoglein (g). Note that the vesicles with electron-dense plaques are heavily decorated with the desmoplakin antibodies (e and f) as well as, more weakly in this experiment, with anti-desmoglein (arrows in g). Bars, 0.2 μm.



peared in dots as visualized by immunofluorescence microscopy. For example, human A-431 cells grown in LCM for several months and serially passaged using trypsin-EDTA buffer always contained numerous dots positively immunostained for desmoplakin, desmoglein, and plakoglobin, and many of these dots seemed to be aligned on cytokeratin IF bundles (Fig. 5, *a* and *b*). Again, double-label immunofluorescence microscopy showed most of the dots positive for both the transmembrane glycoprotein marker, desmoglein, and the plaque proteins, desmoplakin and plakoglobin (not shown; for details see 14).

When examined by electron microscopy, these cells always contained a high number of intracellular vesicles with plaques and IF bundles attached (Fig. 5 *c*). In addition, they showed variously sized plasma membrane domains equivalent to "half-desmosomes", with dense plaques and IFs attached (Fig. 5, *d-g*), occasionally even showing the periodical small surface projections resembling those revealed in the desmosomal midline material (arrow in Fig. 5 *d*; cf. 9, 19, 61). Such half-desmosomal structures were not only seen in regions where cells approached each other (Fig. 5 *d*) and at the ventral side but also on the upper surfaces of cells (Fig. 5, *e-g*). They occurred on exposed ("free") cell surface regions (Fig. 5, *e-g*) as well as in invaginations that were in continuity with surface membrane caveolae (Fig. 5 *h*). Occasionally, "accessory plaque" structures (Fig. 5 *g*), similar to those previously described in certain bovine cell cultures kept in NCM (9, 57), were also noted.

Immunoelectron microscopy, notably double-label experiments, of LCM-grown A-431 cells identified both, cytoplasmic vesicles (Fig. 6 *a*) and half-desmosome equivalents (Fig. 6 *b*), as positive for all three markers, i.e., desmoplakin, plakoglobin, and desmoglein. Vesicles with plaques positive for desmosomal markers included some "multivesicular bodies" (Fig. 6 *a*), which might indicate the occurrence of lysosomal degradation of at least some desmosomal structures. Extended cell surface regions in which several relatively thin inner plaques were covered by a common thicker but looser, IF-interwoven plaque with positive desmoplakin label were also observed (Fig. 6 *c*) and might represent fused half-desmosomes (for fused desmosomes see, e.g., 9).

Upon shift to NCM, A-431 cells also formed desmosomes, and this process was accompanied by a gradual reduction of a moderate cytoplasmic background desmoplakin immunoreactivity (data not shown).

Biochemical Studies

Biosynthesis of Desmosomal Proteins in LCM. To examine whether the major desmosomal proteins studied were con-

tinually synthesized under LCM conditions we metabolically labeled murine keratinocytes grown for two weeks in LCM with [³⁵S]methionine and immunoprecipitated, from total cell lysates, desmoplakin (Fig. 7 *a*, lane 1, and *b*), desmoglein (Fig. 7 *a*, lanes 2 and 3) and plakoglobin (Fig. 7 *a*, lane 5). These results showed that all three proteins were synthesized in LCM cultures. This was also found in bovine (MDBK) and human (A-431) cell cultures (see also below), confirming the results of Penn et al. (52) for desmoplakin and desmoglein. The LCM-grown mouse keratinocytes synthesized both desmoplakins I and II (Fig. 7 *a*, lane 1, and *b*) as well as the basic "band 6 polypeptide" (Fig. 7 *a*, lane 6), reflecting their derivation from a stratified epithelium. Identification of the same polypeptides metabolically labeled with [³⁵S]methionine after the shift to NCM is shown in Fig. 7 *a*, lanes 8-14.

Distribution and State of Desmosomal Plaque Proteins in LCM. The major desmosomal proteins characterized so far, including plaque and membrane proteins, are known for their insolubility in buffers of physiological ionic strength and pH and are resistant to the action of nondenaturing detergents. They are also relatively resistant to extraction treatments with buffers of very low and high ionic strengths as well as very low and high pH values (pH ~2.3-9.0; 13, 17, 19, 25, 59). Only for plakoglobin, a significant proportion has been recovered in a soluble, apparently dimeric form of ~7S from supernatant fractions of various epithelial and non-epithelial cells (11, 33-35). Remarkably, in cell fractionation studies of the distribution of desmoplakin in structure-bound (pelletable) and supernatant (soluble) fractions from mouse keratinocytes grown for 2 or 3 wk in LCM we found most of it (≥90%) in a soluble form (Fig. 8 *a*, *b* and *b'*). Unexpectedly, however, the relative amount of soluble plakoglobin, which is between 20 and 30% of total plakoglobin in tissues and NCM-grown cultures of various sources, including keratinocytes (data not shown; cf. 33-35), did not considerably increase in LCM (Fig. 8, *a* and *c*).

Examination of the physical state of the LCM-soluble desmoplakin by sucrose gradient centrifugation (Fig. 8, *d* and *e*) revealed a monodisperse distribution with a peak fraction sedimenting faster than rabbit IgG and slower than catalase, corresponding to ~9S. This indicates that the soluble desmoplakin predominant in LCM-grown keratinocytes exists in a distinct molecular form, either as a monomer or as a complex (dimer or heterotypic).

When we examined the existence of a pool of soluble desmoplakin in LCM-treated murine keratinocytes in other cells such as in bovine MDBK (data not shown; cf. 14) and human A-431 (Fig. 9) cells, we observed essentially the same. For example, the result of a typical fractionation experiment in

Figure 5. Organization of cytoskeletal and desmosomal proteins in human carcinoma (A-431) cells continuously passaged in LCM and, 3 d after plating, fixed with methanol and acetone, for immunofluorescence microscopy (*a* and *b*), or with glutaraldehyde and OsO₄ for electron microscopy (*c-h*). (*a* and *b*) Double-label immunofluorescence with anti-cytokeratin (*a*) and anti-desmoplakin (*b*). Note extended cytokeratin IF arrays and numerous cytoplasmic dots; many dots seem to be aligned on IF bundles (arrowheads point to some examples). (*c-h*) Electron microscopy of plaque-bearing membrane domains, including vesicles with desmosomal plaques and attached tonofibrils (*T*) (*c*, arrow denotes plaque) and "half-desmosomes" on the plasma membrane displaying typical desmosomal features (*d-h*; brackets denote plaques; arrow in *d*) projections resembling desmosomal midline structures). (*CV*) coated vesicle. Half-desmosomal domains occur on free plasma membrane regions (*e-h*) as well as in regions of cell contact (*d*). Some half-desmosomes are found to be continuous with surface caveolae (big arrowheads in *h*; small arrowheads denote plaque-connecting IF bundle; arrows denote plaques). The brackets in (*g*) demarcate the primary and the "accessory" plaque. Bars, (*a* and *b*) 20 μm; (*c-h*) 0.2 μm.

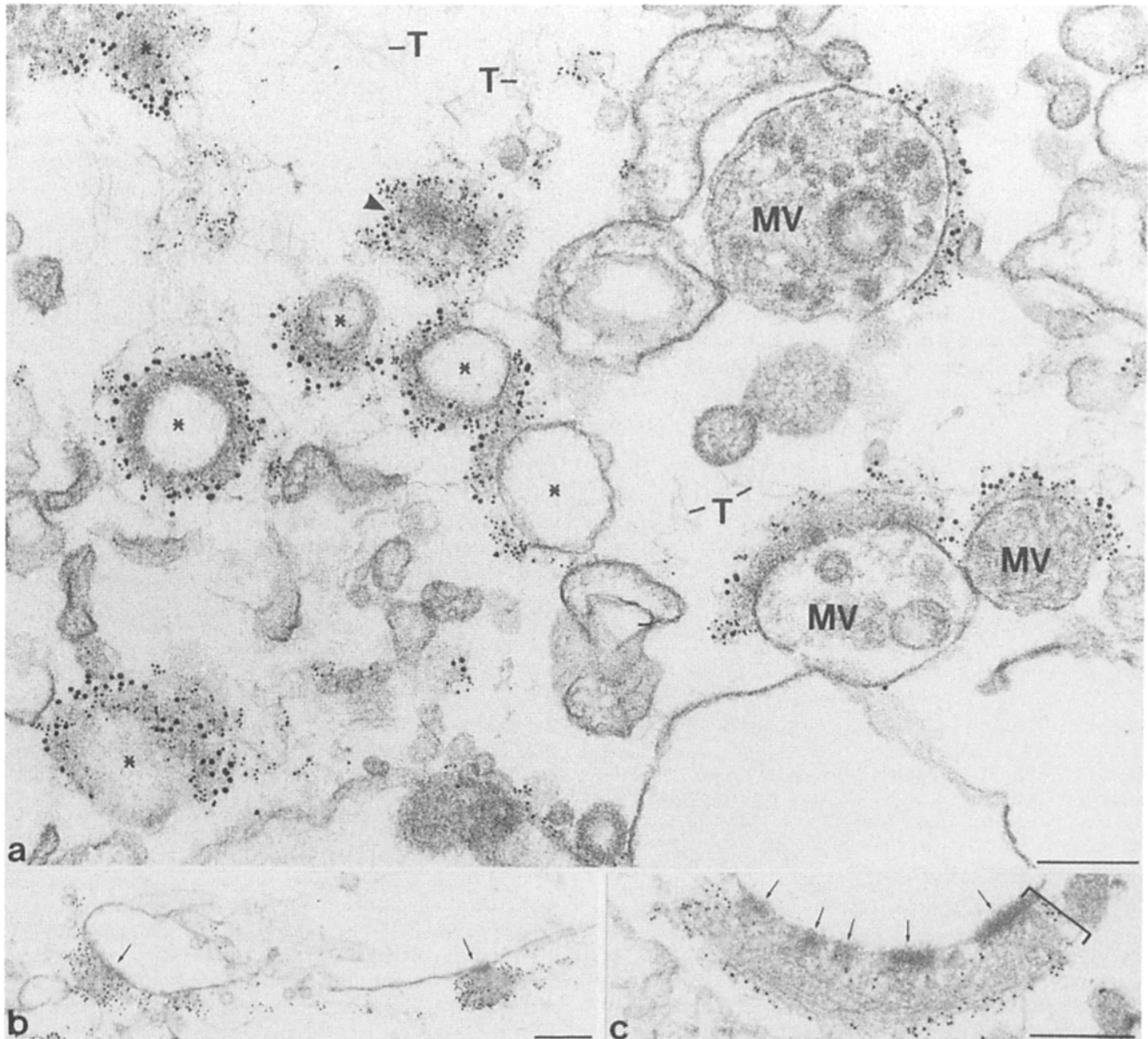


Figure 6. Electron microscopic immunolocalization of desmosomal proteins in LCM-grown A-431 cells (same as in Fig. 5). Cells grown on poly-L-lysine-coated coverslips were lysed with 0.01% saponin in PBS and processed as described in Materials and Methods. (a) Double-label localization of plakoglobin and desmoglein with guinea pig anti-plakoglobin (10-nm gold) and murine desmoglein anti-body DG3.10 (5-nm gold). Vesicles with electron-dense plaques (asterisks) and plaque-attached IF bundles, i.e., tonofibrils (T), are recognized. Structures positively decorated for desmosomal proteins but without a visible membrane (an example is denoted by the arrowhead) probably represent section planes grazing to vesicle surfaces. (MV) Multivesicular bodies. (b and c) Half-desmosome plaques on the plasma membrane (arrows) positively decorated with anti-desmoplakin (5-nm gold) can appear as individual domains (b) or integrated into a larger region covered by a thick mat of plaque material and IF structures (bracket in c). Bars, 0.2 μ m.

A-431 cells is shown in Fig. 9, a-d. While most of the desmoplakin was consistently recovered in the supernatant fraction (70-90% of the total desmoplakin I; Fig. 9 b) desmoglein was distributed over all fractions, apparently due to its partial solubilization with saponin (Fig. 9 c), and the percentage of soluble plakoglobin (Fig. 9 d) was again similar (i.e. \sim 30%) to that found in NCM-grown cells of different kinds. In the interpretations of the different LCM behavior of plakoglobin, in comparison with desmoplakin, it should be kept in mind that plakoglobin but not desmoplakin also exists in other, i.e., nondesmosomal plaque structures (cf.

11, 34, 35; for different behavior of nondesmosomal adhering junctions during Ca^{2+} depletion and shift to NCM see references 36, 41, 42, 47).

Discussion

Under LCM conditions preventing the formation of desmosomes, cell cultures continue to synthesize all major desmosomal components, those exclusive to the cytoplasmic plaque as well as transmembrane glycoproteins (this study and 52; see, however, 41). Ben Ze'ev's (1) report of a de-

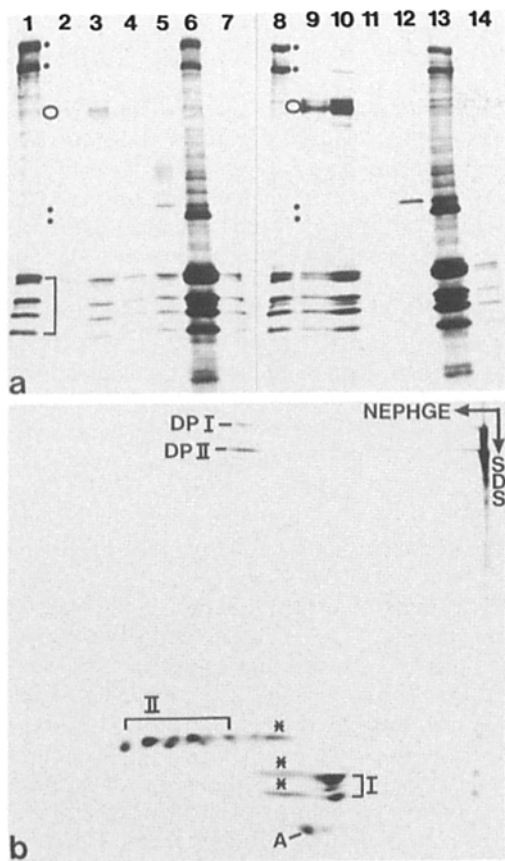


Figure 7. Synthesis of desmosomal proteins in primary mouse keratinocytes grown for 2 wk in LCM. Cells of the same primary culture were labeled in LCM (lanes 1-7) or 6 h after shift to NCM (lanes 8-14) with [³⁵S]methionine, and desmosomal proteins were immunoprecipitated from total cell lysates, using the following antibodies: (Lanes 1 and 8) Anti-desmoplakin; (lanes 2 and 9) monoclonal antibody to desmoglein (DG3.10); (lanes 3 and 10) guinea pig antibodies to desmoglein; (lanes 4 and 11) negative controls (irrelevant guinea pig antiserum); (lanes 5 and 12) monoclonal antibody PG5.1 to plakoglobin; (lanes 6 and 13) guinea pig antiserum reactive with several desmosomal proteins, including desmoplakins, plakoglobin, and "band 6 polypeptide"; (lanes 7 and 14) negative controls (protein A-Sepharose alone). Dots in lanes 1 and 8 denote, from top to bottom, the positions of desmoplakin I and II, plakoglobin and "band 6 polypeptide" of bovine snout epidermal desmosomes. The open circle indicates the position of desmoglein. The bracket in the lower part of the gel denotes cyokeratin polypeptides that have reconstituted filaments and were nonspecifically precipitated under the conditions chosen here for improvement of yields of desmosomal proteins (see also negative controls in lanes 4, 7, 11 and 14). (b) Autoradiofluorograph of an immunoprecipitate with anti-desmoplakin (same sample as in a, lane 1) after two-dimensional gel electrophoresis (nonequilibrium pH gradient electrophoresis, NEPHGE, was used in the first and SDS-PAGE in the second dimension; basic polypeptides to the left). Both desmoplakin I and II (DP I and DP II) are synthesized in these cells. In the lower part of the gel the cyokeratins (I, acidic type I and II, basic type II polypeptides) are seen (type II components have M_r values of 58,000 and 59,000, type I components M_r ~ 53,000 and 49,000; for details of cyokeratin expression in murine LCM grown keratinocytes see references 4 and 54). Asterisks mark cyokeratin complexes not denatured during electrophoresis in the first dimension. (A) Actin.

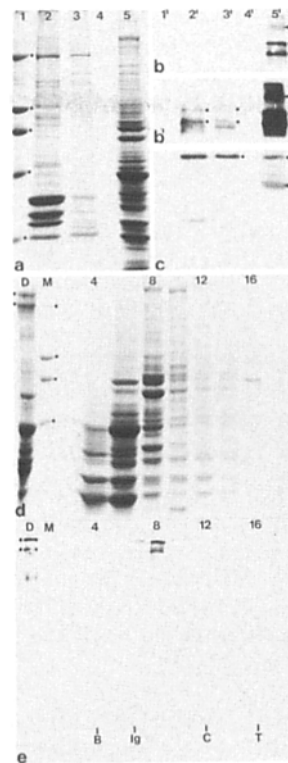


Figure 8. Fractionation and characterization of soluble and pelletable forms of desmosomal plaque proteins in LCM-grown primary mouse keratinocytes. (a-c) Primary mouse keratinocytes grown for 2 wk in LCM were homogenized in near-physiological salt buffer and the lysate fractionated as described in Materials and Methods. (a) Coomassie Blue-stained gel. (Lane 1) Molecular mass reference proteins (Dots denote, from top to bottom: 220,000; 116,000; 92,000; 68,000; 45,000). Lanes 2 and 3 contain the low and high speed pellets of the homogenate, respectively. (Lane 4) blank. (Lane 5) 100,000 g supernatant. (b, b', and c) Autoradiographs showing immunoblot reactions corresponding to lanes 1-5. Horizontal strips from the same nitrocellulose filter paper sheet were incubated with different antibodies. (b and b') antibodies to desmoplakin (b' shows a longer exposure of b), (c) plakoglobin antibody. Note that the vast majority of desmoplakin is found in the 100,000 g supernatant (lane 5') whereas

only minor portions, with some enrichment of a component corresponding to desmoplakin II, are recovered in the pellet fractions (2' and 3'). In (b and b') the dots denote the positions of desmoplakins I and II, the arrowhead points to cross-linked desmoplakin not cleaved under the SDS-PAGE conditions used. Note that plakoglobin (dot in c) is recovered, at nearly equal proportions, in all fractions (arrowhead in c denotes proteolytic breakdown product of plakoglobin; cf. reference 45). (d and e) Characterization of soluble desmoplakin recovered in the 100,000 g supernatant fraction by centrifugation in a 5-30% (wt/vol) sucrose gradient and analysis of the fractions by SDS-PAGE and immunoblotting. (d) Coomassie Blue-stained gel. (Lane D) Proteins of a crude desmosomal fraction from bovine snout epidermis (dots denote desmoplakin I and II). (Lane M) Molecular mass reference proteins (Dots denote, from top to bottom: 220,000; 116,000; 92,000; 68,000). Fraction numbers (only even numbers are indicated) are given at the top margin. (e) Immunoblot with anti-desmoplakin of a gel with identical loading to that shown in (d). Note that the peak fraction of desmoplakin (no. 8) sediments between rabbit IgG and catalase with an estimated sedimentation value of about 9S. The relative positions of marker proteins are indicated: (B) BSA (4.3S); (Ig) rabbit IgG (7.0S); (C) catalase (11.15S); (T) thyroglobulin (16.5S).

creased synthesis of desmoplakin in MDBK cell cultures in which cell contacts had been split with 12-o-tetradecanoylphorbol-13-acetate (TPA) is not relevant in this respect as his analysis was restricted to cytoskeletal fractions and soluble proteins were excluded. Clearly, the present study and that of Penn et al. (52) shows that the formation of desmosomes or other stable cell-cell contacts is not required for the maintenance of synthesis of desmosomal proteins, although intercellular contacts may result in modulations of the specific rates of synthesis and turnover of some desmosomal components, especially desmoglein (see Fig. 7 a).

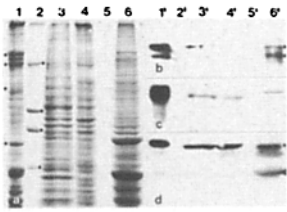


Figure 9. Identification of soluble and pelletable forms of desmosomal plaque proteins in cell homogenates from LCM-grown A-431 cells. (a) Coomassie Blue-stained gel. (Lane 1) contains crude desmosomal fraction from bovine snout epidermis (Dots denote, from top to bottom: desmoplakin I and II, desmoglein

and plakoglobin). (Lane 2) Molecular mass reference proteins (as in Fig. 8 d). Lanes 3 and 4 contain the low and high speed pellets, respectively. (Lane 5) Blank. Lane 6, total protein of 100,000 g supernatant fraction. Lanes 1'-6' show autoradiographs of immunoblots corresponding to lanes 1-6 (strips from the same sheet were processed as described for Fig. 8, b-c), using antibodies to desmoplakin (b), desmoglein (c) and plakoglobin (d), to identify all three proteins in the same experiment. Note that most of the desmoplakins I and II (denoted by the dots in b) are found in the 100,000 g supernatant (only small amounts of mostly desmoplakin I are detected in lane 3', denoted by small arrow, which happens to be concentrated on one side of the lane in this experiment), whereas desmoglein (c) and plakoglobin (d, designations as in Fig. 8 c) are recovered in comparable proportions in all fractions.

Our results show that in LCM, most—in murine keratinocytes perhaps all—desmoplakin exists in a monodisperse, soluble form distributed over the cytoplasm. They also indicate that, at least in murine keratinocytes, new desmosomes emerge rapidly, i.e., in an interval between 5–20 min upon shift to NCM, directly at the cell surface, i.e., without detectable intermediate aggregate forms of desmoplakin on IF bundles deep in the cytoplasm, as it has been proposed by Goldman and colleagues (27, 32).

When we compare our immunofluorescence and immunoelectron microscopic results with those of Jones and Goldman (32) we suspect that many, perhaps all of their “desmoplakin aggregates” on cytoplasmic, particularly perinuclear IF bundles are vesicle-associated and that many, probably all, of them are endocytosed residues of desmosomes split during dissociation of the tissue (cf. 36, 37, 49). We are aware, however, that our results do not exclude the possibility that some of these vesicles may be derived from newly formed (unstable) desmosomes or from half-desmosomes of the kind found in A-431 cells. The vesicular association of the desmoplakin-containing dense plaques, with IFs attached, may easily be overlooked in individual ultrathin sections. However, as we show in the present study, the vesicle membrane is readily demonstrable in grazing and serial sections (cf. 36) as well as with markers of integral membrane proteins such as desmoglein. We also show that long periods of culturing in LCM are required to clear the cytoplasm completely from such desmosome-derived vesicles formed as the result of trypsination and/or Ca^{2+} depletion by chelation or by exposure to LCM. Obviously, 4 d of LCM culture are not sufficient to deplete keratinocytes from such desmosomal plaque-bearing vesicles (for related observations in MDBK and MDCK cells see also references 41 and 42). Therefore, in studies of the junction formation the longevity of such internalized domains should not be underestimated. Of course, the data presently available still leave the possibility that some of the desmoplakin-positive dots might indeed be aggregates of plaque material without a vesicle, or that some

plaque-bearing vesicles may contribute to the formation of desmosomes by exocytosis, although we see no evidence for this process.

Several reasons may have contributed to the discrepant results of previous authors describing desmoplakin immunostaining in the cytoplasm of LCM-grown cells who have reported only weak, diffuse reaction in human keratinocytes (64) or no diffuse but distinct “dotty” reaction in murine keratinocytes (32) or in MDBK and MDCK cells (41). For example, losses of soluble desmoplakin during immunolocalization preparations involving partial cell lysis, repeated washes, and prolonged incubations in buffers (cf. 38), may be responsible for an artificial reduction of the fluorescence of soluble proteins. Some of the differences could also be due to the different periods of time after LCM shift in the specific experiment. In addition, there seems to be a widespread tendency to adjust the illumination conditions to the detection of strong signals, in these cases punctate arrays, and to consider weak diffuse immunostaining as unspecific background noise.

Interpretations of morphological studies of the state of junctional components in LCM and of the formation of desmosomes upon shift to NCM are obviously even more complicated by our finding that, even in LCM, certain cells such as human A-431 cells are able to assemble desmosome-type plaques on desmoglein-containing membrane domains, resulting in the formation of half-desmosome equivalent structures on plasma membrane regions, which then may become endocytosed and end up as cytoplasmic vesicles. The mere existence of this phenomenon, which may be much more common than hitherto thought (see also references 44, 57, 58), shows that both the clustering of desmosomal membrane components (cf. 8) and the assembly of plaque proteins are not necessarily coupled to the formation of stable cell junctions. Therefore, cultures such as LCM-grown A-431 cells may provide an experimental system to separate processes of cell-cell junction formation from those involved in the assembly of proteins characteristic of the desmosomal complex. Similar small asymmetric desmosomal structures have also been described as possible intermediates of desmosome formation in studies of cell cultures growing in NCM (12) and during LCM-NCM shift (28), as well as in early embryogenesis (cf. 31) and certain adult tissues (for review see reference 9). Hence, it may be possible that such half-desmosomal complexes at the cell surface are recruited, upon shift to NCM, for the formation of complete desmosomes.

We interpret the recovery of most desmoplakin in supernatant fractions from LCM-grown cells lysed in buffers of near-physiological ionic strength and pH primarily to represent protein existing in this form within the living cell, in agreement with the dispersed immunofluorescence. The finding of a large proportion of soluble desmoplakin, even exceeding the relative amounts of soluble plakoglobin (33), is astonishing as desmoplakin in its desmosome-bound form is notoriously insoluble. Small pools of distinct soluble forms have recently also been described for various cytoskeletal systems, IF proteins included (e.g., 21, 60). From preliminary analyses of the state of desmoplakin in NCM-grown cells (not shown; cf. 14) we conclude that a certain small proportion of desmoplakin also exists in a soluble cytoplasmic state in desmosome-connected cells although it is technically

difficult to quantitate this pool. At present we do not know whether this small soluble pool in NCM-grown cells represents a special kinetic fraction, e.g., newly synthesized molecules, or a specifically modified form of the protein. In view of the reports that the soluble forms of other cyto- or karyoskeletal proteins such as nuclear lamins, vimentin, and desmin might be regulated by specific phosphorylation events (e.g., 23, 30, 48), it is worth mentioning that desmoplakins are, to a high degree, phosphorylated (46) and that the carboxy terminal amino acid sequence of desmoplakins contains clusters of oligopeptide repeats rich in arginine, glycine, and serine which could provide good substrate sites for certain protein kinases (Franke, W. W., H. Mueller, and P. Cowin, unpublished data). Because of the possibility that Ca^{2+} itself may be involved in the regulation of desmoplakins phosphorylation and/or dephosphorylation we mention the calmodulin-binding protein, desmocollin, which is enriched at the desmosomal plaque of certain bovine epithelia (63). The obvious effect of Ca^{2+} on the assembly of the desmosomal plaque proteins could either be direct, e.g., by Ca^{2+} binding to desmoplakins, or indirect through another Ca^{2+} -binding component. Among the possible candidates for the latter function one has to mention both kinds of major desmosomal glycoproteins, desmoglein, and the two desmocollins, which have been reported to bind calcium ions (43, 62).

Although it is obvious that desmosomes, through their anchorage of IF bundles, provide a major principle of IF architectural organization our results with LCM-grown cells of diverse kinds such as mouse keratinocytes, human A-431 cells and bovine MDBK cells show that extended IF bundle arrays, approaching the cell cortex, can also be formed and maintained in the absence of desmosomes (see, e.g., Figs. 2 b and 5 a). This conclusion, which is at variance with other authors' concepts of a mutual dependence of IF formation and/or organization on the one hand and desmosome formation on the other (e.g., 3, 32, 64), is supported by the existence of certain cells such as rat kangaroo PtK₂ which possess massive meshworks of cytokeratin IF bundles in the absence of true desmosomes and of desmoplakins (15, 17, 18; for review see reference 9).

After completion of the present study (cf. 14) a bipartite publication has appeared (50, 51) in which the biochemical and morphological state of desmoplakins in LCM-grown MDCK cells has been studied. These authors' biochemical data are compatible with ours in that they show desmoplakins synthesis in LCM and a special pool of desmoplakins that is soluble in Triton X-100-high salt buffer (however, to a lesser degree, i.e., 65%) in a monodisperse form (reported, however, to sediment at 7.35S) although their use of an extraction medium of high ionic strength and relative high Mg^{2+} concentration (3 mM) does not allow a direct comparison with our results. We also agree that the diffuse cytoplasmic desmoplakins immunostaining described for LCM cells by these authors reflects the soluble pool of desmoplakins, as in our study. However, their interpretation of immunofluorescent cytoplasmic dots as precursor structures of desmosome formation, which is essentially identical with that of Jones and Goldman (32), is at variance with our results and those of others (42). Obviously Pasdar and Nelson (50, 51) dismiss the possibility of the existence of intracellular vesicles and half-desmosomes on the cell surface, i.e., states of desmosomal material that have been demonstrated in these and

other cell cultures at the times used for LCM exposure in their study, i.e., from 12 h to 2.5 d. Moreover, in contrast to these authors we have no difficulties in detecting the preservation of numerous desmoplakins dots in the cytoplasm of cells fixed 9 h after LCM-NCM shift (e.g., Fig. 3 D of reference 51).

Our observations suggest that the assembly of desmosomal plaques and desmosomes upon shift from LCM to NCM does not require the formation of large plaque precursor aggregates on IFs in the cytoplasm and the subsequent movement of these preformed large plaque-IF bundle complexes from the interior to distinct sites at the plasma membrane but occurs by the direct assembly of desmoplakins and plakoglobin from pools of diffusible proteins which is nucleated at certain membrane domains. The latter in turn, may be provided by the clustering of integral membrane components such as desmoglein. This membrane domain-nucleated assembly of proteins from a soluble pool would allow a fast, effective and site-targeted formation of desmosomes, independent from the amounts and patterns of distribution of IF bundles. Considering the speed with which IF protein assembly can generally occur we see no difficulty assuming that these nascent plaques are immediately active in nucleating IF protein assembly and IF bundle anchorage.

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