

# Continual Assembly of Half-Desmosomal Structures in the Absence of Cell Contacts and Their Frustrated Endocytosis: A Coordinated Sisyphus Cycle

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**Abstract.** It is widely assumed that the coordinate assembly of desmosomal cadherins and plaque proteins into desmosome-typical plaque-coated membrane domains, capable of anchoring intermediate-sized filaments (IF), requires cell-to-cell contacts and a critical extracellular  $\text{Ca}^{2+}$  concentration. To test this hypothesis we studied several cell lines grown for years in media with less than 0.1 mM  $\text{Ca}^{2+}$  to steady-state low  $\text{Ca}^{2+}$  medium (LCM) conditions, particularly the human keratinocyte line HaCaT devoid of any junctional cell contact (HaCaT-L cells). Using immunolocalization and vesicle fractionation techniques, we found that the transmembrane glycoprotein, desmoglein (Dsg), colocalized with the plaque proteins, desmoplakin and plakoglobin. The sites of coassembly of desmosomal molecules in HaCaT-L cells as well as in HaCaT cells directly brought into LCM were identified as asymmetric plaque-coated plasma membrane domains (half-desmosomes) or as special plaque-associated cytoplasmic vesicles, most of which had formed endocytotically.

The surface exposure of Dsg in these half-desmosomes was demonstrated by the binding, in vivo, of antibodies specific for an extracellular Dsg segment which also could cross-bridge them into symmetric quasi-desmosomes. Otherwise, these half-desmosomes were shown in LCM to be taken up endocytotically. Half-desmosomal assemblies were also seen in uncoupled cells in normal  $\text{Ca}^{2+}$  medium. We conclude that, in the absence of intercellular contacts, assembly of desmosomal proteins at the cell surface takes place, resulting in transient half-desmosomes which then, in LCM and without a stable partner connection to the adjacent cell, can be endocytotically resumed. This frustrated cycle of synthesis and assembly maintains an ensemble of molecules characteristic of epithelial differentiation and the potential to form desmosomes, even when the final junctional structure cannot be formed. We propose that these half-desmosomal structures are general cell structures of epithelial and other desmosome-forming cells.

**A**DHERING junctions are cell-connecting structures that form at a critical  $\text{Ca}^{2+}$  concentration and are characterized by closely aligned plasma membrane domains, with 20–30 nm lipid bilayer equidistance, and an electron-dense cytoplasmic plaque anchoring bundles of cytoskeletal filaments. Two major kinds of adhering junctions have been distinguished according to morphological criteria and the specific type of filaments attached (14, 22, 77): Adherens junctions such as the *zonula* or *fascia adherens* are associated with actin microfilaments whereas intermediate-sized filaments (IFs)<sup>1</sup> are attached to desmo-

somes (*maculae adherentes*). Extensive analyses of the molecular composition of adhering junctions from diverse tissues and cell culture lines have revealed common principles such as the presence of clustered transmembrane glycoproteins of the cadherin family of cell adhesion molecules and the existence of a common plaque protein, plakoglobin, but also extensive differences (15, 44; for recent reviews see references 26, 43). In addition, more recent analyses have shown that further subtypes of adhering junctions can be defined by their specific complement of cadherins and plaque proteins (for review see reference 75).

IF bundles are anchored at desmosomal plaques formed by the cytoplasmic portions (tails) of one or more representatives of either subfamily of desmosomal cadherins, the desmocollins (Dsc1-3) and the desmogleins (Dsg1-3; for nomenclature see reference 11), in association with special cytoplasmic plaque proteins, including plakoglobin and desmoplakin I (24, 25, 28). In addition, desmosomal

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1. *Abbreviations used in this paper:* Dsc, desmocollin; Dsg, desmoglein; HaCaT-L, human keratinocyte cell culture line HaCaT adapted to LCM by long term culturing; IF, intermediate-sized filament(s); LCM, low  $\text{Ca}^{2+}$  medium; MVB, multivesicular body; NCM, normal  $\text{Ca}^{2+}$  medium.

plaques can contain certain cell type-specific accessory proteins such as desmoplakin II, antigen 8L, or plakophilin 1 (band 6 protein), and the pattern of these plaque proteins together with the specific complement of Dsg and Dsc isoforms defines the subtype of desmosome present in a given cell (26, 31, 43, 47, 54, 70, 71, 75).

The  $\text{Ca}^{2+}$  dependence of the formation and maintenance of desmosomes is particularly clear from observations that the coherence of cells grown in monolayer culture can be disrupted by the reduction of extracellular  $\text{Ca}^{2+}$  concentration below 0.1 mM either by the addition of  $\text{Ca}^{2+}$  chelators or by transferring the cells to low  $\text{Ca}^{2+}$  medium (LCM). This cell dissociation by  $\text{Ca}^{2+}$  depletion has been demonstrated in a diversity of cell types, including epithelial cells such as primary keratinocytes (21, 32, 33, 37, 56, 86, 87, 90) and various cell lines derived from stratified or simple epithelia (e.g., references 12, 21, 27, 29, 39, 42, 50, 51, 60–62, 66–68) as well as myocardial cells (40). The splitting of desmosomes in LCM leads to rapid endocytosis of the resulting half-desmosome structures and then in the accumulation of cytoplasmic vesicles associated with plaques and IF bundles (e.g., references 21, 34, 39–42, 50), similar to the structures endocytotically formed after the disruption of desmosomes by proteases (e.g., references 57, 58, 39 and references therein). The fate of such desmosome-derived endocytotic vesicles is apparently not uniform as some of them can be seen in secondary forms of vesicles related to the lysosomal degradative compartment (e.g., references 10, 21) whereas other vesicles seem to belong to a nondegradative compartment (34).

Biochemical studies of the synthesis and physical states of desmosomal proteins and glycoproteins in cells grown in LCM for a limited period of time have demonstrated the continued synthesis and processing of Dsg and Dsc and the plaque proteins, in certain cells with an increased cytoplasmic pool of a soluble distinct 7.3-9 S form of desmoplakin (8, 20, 21, 37, 60-62, 64, 66–68). Controversy, however, exists as to the dependence of the coassembly of desmosomal cadherins with the cytoplasmic plaque proteins, notably desmoplakin, on extracellular  $\text{Ca}^{2+}$  and on cell contact formation, particularly with respect to the mechanisms of  $\text{Ca}^{2+}$ -mediated assembly of desmosomal structures at the cell surface. For example, it has been reported by some authors that in LCM-grown cells the insoluble forms of desmoplakin and the transmembrane glycoprotein, Dsg, were not associated with each other but differently located (37, 64) and that Dsc, Dsg and desmoplakin would only associate with each other and assemble into plaque structures upon induction of stable cell-cell contacts at normal  $\text{Ca}^{2+}$  concentrations (61, 62, 64, 65; see also 37, 51, 52, 68, 87). On the other hand, the regular formation of plaque-bearing, half-desmosomal structures containing Dsg and desmoplakin at the plasma membrane and on vesicles has been noted in several LCM-grown cell cultures (20, 21).

In the present study, we have followed up our observation of the assembly of desmosomal proteins in the absence of cell contacts and have grown cell lines for several years continuously in LCM to provide well-defined steady state conditions and to avoid possible disturbances of short term LCM adaptation. Using the human keratinocyte reference line HaCaT, we show that in such LCM-adapted

cells (HaCaT-L cells) not only the synthesis of all desmosomal components is continued but that the coassembly of desmosomal cadherins and plaque proteins into desmosome-like plaque structures (half-desmosomes), as well as IF anchorage, can also take place in the absence of stable cell contacts. This suggests that the critical  $\text{Ca}^{2+}$  concentration  $>0.1$  mM is not required for the coassembly of desmosomal cadherins and plaque proteins at distinct plasma membrane sites but only for the last step of desmosome formation, i.e., the induction and stabilization of the symmetrical junction at the cell surface.

## Materials and Methods

### Cell Culture

Cultured human keratinocytes of the permanently growing non-malignant line HaCaT, capable of reforming normally differentiated epidermis upon transplantation onto the dermis of nude mice (7), were grown in 100 mm plastic dishes (Falcon, Oxnard, CA) and on poly-L-lysine-coated glass coverslips (0.01% poly-L-lysine in PBS) in normal (NCM) or LCM  $\text{Ca}^{2+}$  medium (60–90  $\mu\text{M}$  Ca) consisting of DME without  $\text{Ca}^{2+}$  (Biocrom, Berlin, FRG) supplemented with 10%  $\text{Ca}^{2+}$ -depleted FCS (21). The  $\text{Ca}^{2+}$  concentration of the FCS was reduced below 0.1 mM by passing it over a Chelex 100 column (Bio-Rad Laboratories, München, FRG), and the actual  $\text{Ca}^{2+}$  concentration was determined by atom absorption spectroscopy. In the experiments described here, HaCaT cells growing continually in LCM for 5 yr were used (HaCaT-L cells).

For most microscopical experiments, cells were allowed after plating to grow for 3 d, whereas for most biochemical experiments cells were harvested after 7 d, with a medium change after the third day. The distribution pattern of Dsg or desmoplakin was not considerably altered between 3 and 7 d of LCM-culture. In special control experiments, cells plated at very low density and widely distant from each other were examined by immunofluorescence and electron microscopy. Other human and bovine cell lines grown as described (e.g., references 12, 21, 74) were examined with the same methods.

Shifts from LCM to NCM have been described for various cells (20, 21). Specifically, during this study LCM-adapted cells were once washed with NCM, plated at very low cell density in NCM, and examined at 24 or 48 h. Alternatively, NCM-grown cells were transferred to, and grown in, LCM for one to two passages, i.e., up to 2 wk.

### Antibodies

The following monoclonal murine antibodies against desmosomal proteins were used: (a) Antibodies DP 2.15, 2.17, 2.19, 2.20 against desmoplakin (13) were used either separately or as a mixture (DP-mix; available as multi-epitope cocktail from Progen Biotechnik, Heidelberg, FRG); (b) antibodies DG 3.10 and 3.4 against desmoglein (72, 73); (c) plakoglobin antibodies PG5.1 (15; from Progen) and 11E4 (kindly provided by Dr. M. Wheelock, University of Toledo, OH), and (d) antibodies Dsg2E-G11 and Dsg2E-G129 against the extracellular part of Dsg2 (71; Demlehner, M. P., S. Schäfer, C. Grund, and W. W. Franke, unpublished results).

mAb RCK 102 from EuroDiagnostics via LaboServ (Giessen, FRG) recognize cytokeratins 5 and 8, mAb OD 5.9 (from Progen) was raised against the heavy chain of clathrin (9), and mAb H4A3 reactive with human lysosomal membrane glycoprotein 1 (49) was obtained from the Developmental Studies Hybridoma Bank (Johns Hopkins University School of Medicine, Baltimore, MD).

Rabbit antibodies against bovine desmoplakin were a generous gift from Dr. J. W. Nelson (Stanford University of Medicine, Stanford, CA) and guinea pig antibodies against bovine desmoplakins I and II and other desmosomal proteins were prepared as described (see references 23, 24). Guinea pig and rabbit antibodies against different parts of human desmoglein Dsg2 were obtained by immunization with partial polypeptides synthesized in *Escherichia coli*, using the his-tag technique and pQE-expression vectors of Diagen (Düsseldorf, FRG), followed by purification from bacterial lysates on a nickel-chelate affinity column.

Guinea pig antibodies (Dsg2-E2/E3) against an extracellular portion including domains E2-E3 of human Dsg2 were raised by immunizing the animals with partial protein obtained by cloning a EcoRV/HindIII frag-

ment of clone hDsg2.zk16 (nucleotides 461–1249; reference 71) into plasmid pQE32, digestion with restriction enzymes SmaI and HindIII, and preparation of the protein from *E. coli* (see above). Rabbit antibodies (DG rab10) against the cytoplasmic tail were raised by using the plasmid HDGC-BB2 (45), in vector pQE9, containing a human Dsg2 DNA-fragment corresponding to nucleotide positions 2340–4237. Rabbit antibodies against desmocollins (DC rab 36) were used for comparison.

### Immunofluorescence Microscopy

Routinely, cells grown on glass coverslips were fixed in methanol and acetone and further processed as described (e.g., references 21, 85). For detection of extracellularly exposed Dsg2 domains, intact cells were incubated in LCM with the primary antibodies for 30 min, 1 or 3 h, then fixed in methanol (5 min) and acetone (30 s) for double-label experiments, or with formaldehyde (5 min) for single-label immunofluorescence microscopy of surface-exposed antigens (see reference 21).

Secondary antibodies coupled to the fluorochromes Texas-Red or FITC (from Dianova, Hamburg, FRG), were used according to the manufacturer's protocol. Primary antibodies were applied undiluted in the case of hybridoma supernatants or diluted 1:80 (guinea pig antibodies) or 1:200 (rabbit antibodies). Cells were usually examined and photographed with a Zeiss Axiophot (Zeiss, Oberkochen, FRG).

### Electron Microscopy

Cells were fixed for 20 min in 2.5% glutaraldehyde in 50 mM sodium cacodylate (pH 7.2), 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 1.25 mM CaCl<sub>2</sub> (4°C), rinsed twice in buffer (4°C), postfixed for 2 h in 2% osmium tetroxide (4°C), block-stained overnight with 0.5% uranyl acetate in H<sub>2</sub>O, and further processed for Epon flat embedding (see reference 85). Vesicular material bound to Dynabeads (see below) was fixed and processed in the same manner. Ultrathin sections were viewed with a Zeiss EM 910 at 80 kV.

For single-label localization of desmoplakin, Dsg or Dsc in HaCaT-L cells, primary antibodies were used for microinjection 1 h after injection the cells were fixed with 2% freshly prepared formaldehyde in PBS for 20 min, permeabilized with 0.1% saponin in PBS for 10 min, incubated for 3–4 h with undiluted gold-coupled secondary antibodies (5- or 10-nm-diam; Amersham Buchler, Braunschweig, FRG) and processed for Epon embedding as described (see above). For double-label immunolocalization cells were gently permeabilized by treatment with 0.01% saponin for 30 s, incubated with antibodies and then fixed and processed as described (21).

Immunolocalization of proteins in sucrose gradient centrifugation fractions was performed by adding the antibodies to the pooled sucrose fractions, using cycling with repeated inversions for 1 h (at 4°C) and a 1:1 dilution with 15 mM Tris-HCl (pH 7.5) and 2 mM DTT. Bound material was collected by centrifugation for 2 h 45 min at 4°C (40,000 rpm, SW40 rotor; Beckman, München, FRG). The pellets obtained were fixed in 2% formaldehyde (in PBS) for 20 min, rinsed with PBS containing 50 mM NH<sub>4</sub>Cl, incubated for 3–4 h (4°C) with the specific secondary antibodies, and then processed for embedding.

For extracellular labeling of cadherins or for studies of endocytosis, living cells grown on glass coverslips were incubated for various periods of time (30 min, 1 h, 3 h) at 37°C in LCM with antibodies to extracellular Dsg2 domains such as mAb Dsg2E-G129 or serum Dsg2-E2/E3, diluted with the solution containing gold-coupled secondary antibodies. Then cells were rinsed with PBS, fixed with buffered glutaraldehyde, postfixed, and then embedded as described. The following control experiments were performed in parallel: (a) formaldehyde-fixed cells were used instead of living ones, without detectable uptake of gold label into the cells; (b) living cells were incubated with antibodies, fixed and then processed with gold-coupled secondary antibodies; (c) cells were exposed to gold-coupled secondary antibodies alone, fixed and further processed.

### Gel Electrophoresis and Immunoblotting

Proteins were separated by SDS-PAGE as described (e.g., reference 1) and visualized by Coomassie Blue or silver (6) staining (the reference proteins for the estimation of apparent kD values were from Bio-Rad Laboratories). Proteins were then transferred to nitrocellulose, and the membranes were treated with 5% fat-free milk powder in blocking buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) for 20 min, primary and secondary antibodies were applied for 1 h in a humid chamber, with extensive washing of the membrane with blocking buffer (3 × 10

min) between each incubation. Antigens were in most cases visualized with the ECL-system (Amersham Buchler), using HRP-conjugated secondary antibodies (dilution 1:5,000–1:10,000). A desmosome-enriched fraction from bovine muzzle epidermis (procedure 3 described of 53) was used for comparison.

### Cell Fractionation and Sucrose Gradient Centrifugation

In all steps precooled solutions (4°C) on crushed ice were used. After rinsing the culture dishes three times with PBS, we added 0.75 ml hypotonic lysis buffer (15 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, and 5 mM DTT) and a special proteinase-inhibitor cocktail containing 2 µg/ml aprotinin, 20 µg/ml leupeptin, 2 µg/ml pepstatin A, 35 µg/ml soybean trypsin-inhibitor (Boehringer Mannheim GmbH, Mannheim, FRG) and 0.1 mM PEFABLOC® SC ([4-(2-aminoethyl)benzenesulfonylfluoride]; Serva, Heidelberg, FRG). The cells were scraped off by a rubber policeman and the cell suspension was treated vigorously five times with a Potter Elvehjem device (Braun, Melsungen, FRG).

The homogenates were centrifuged for 10 min at 800 g (2,500 rpm, A8.24 rotor; Kontron, Neufahrn, FRG) to remove nuclei and other large debris. The supernatant was then again centrifuged for 10 min at 10,000 g (7,500 rpm, SW40 rotor; Beckman), and the postmitochondrial supernatant was fractionated by sucrose gradient centrifugation. 8–15 mg protein were applied to a linear 10–60% (wt/vol) sucrose gradient in 15 mM Tris-HCl (pH 7.5), 2 mM DTT and centrifuged for 12 h (27,000 rpm, SW28 rotor; Beckman). 1.5-ml fractions were collected with a gradient fractionator (Isco, Lincoln, NE) and subjected to protein precipitation (89) or to processing for immunolocalization.

In some cases, the postmitochondrial supernatant of LCM-adapted cells (HaCaT-L cells) was subjected to a flotation gradient by loading 7 ml cell lysate, adjusted to 40% (wt/vol) sucrose, on top of 15 ml 50% (wt/vol) sucrose solution in 15 mM Tris-HCl (pH 7.5), 2 mM DTT, and overlaying these sucrose solutions with 10 ml 33% (wt/vol) and 4 ml 10% sucrose in 15 mM Tris-HCl (pH 7.5) and 2 mM DTT. After centrifugation for 16 h at 25,000 rpm (SW28 rotor), fractions were processed for immunoblotting, or desmoglein-containing vesicles were enriched from the collected peak fractions immunoelectron microscopy (see above). In parallel, tubes were loaded with reference proteins such as catalase and thyroglobulin (11.35S and 16.5S; Pharmacia, Freiburg, FRG) or with *Xenopus laevis* ribosomes (40S, 60S, 80S; for preparation see reference 35).

### Immunoisolation

For immunoisolation of cell structures rich in desmosomal protein(s) from sucrose gradient fractions, magnetic beads (Dynabeads M450; Dynal, Hamburg, FRG) coated with anti-mouse Ig were used. Three adjacent gradient fractions were pooled, diluted 1:1 or 1:3 (at higher sucrose concentration) with immunoisolation buffer (PBS, 0.1% BSA, 1% L- $\alpha$ -phosphatidylcholine) and incubated with mAb DG 3.4 for 1 h. 80 ml Dynabeads, washed twice with immunoisolation buffer, were added to the sample and incubated for another hour, followed by collection of bead-bound proteins in a strong magnetic field (Holder MPC-1; Dynal), three washes with buffer, transfer to a new container, and two final washes. Beads with bound material were either fixed and embedded in Epon for electron microscopy or processed for SDS-PAGE and immunoblotting. For control, sucrose gradient fractions without any added antibodies were mixed with magnetic beads and processed as described.

### Preparation of Whole Cell Lysates and Tissue Extracts

For the preparation of whole cell lysates, cells were scraped off from the culture dish using immunoprecipitation-lysis buffer (15 mM Tris-HCl, pH 7.5, 2% SDS, 8 M urea). The homogenate was shortly sonicated three times (Branson Sonifier B-12; Branson Instrs., Danbury, CT), diluted five times with 15 mM Tris-HCl (pH 7.5), cleared of DNA by methanol/chloroform/H<sub>2</sub>O extraction (89) and then used for SDS-PAGE and immunoblotting.

### Results

To avoid the argument that the previously used malignant cells such as A-431 (see references 20, 21) cells may be anomalously regulated we have focussed on human kerati-

nocytes of line HaCaT that proliferate permanently but are not malignantly transformed, have the potential to differentiate, under appropriate conditions, into an epidermis-equivalent tissue (7) and therefore have become a reference keratinocyte line, whose complement of desmosomal cadherins has been fully determined: Dsg2, Dsg3, Dsc2, and Dsc3 (54, 71).

### **Colocalization of Desmosomal Proteins in Cells Grown in LCM**

The distribution of desmosomal proteins in human HaCaT keratinocytes growing in LCM for several years (HaCaT-L cells) was examined by immunofluorescence microscopy. Cells grown at 60–90  $\mu\text{M}$   $\text{Ca}^{2+}$  were usually separated by a cleft and devoid, even at high cell density, of all kinds of intercellular junctions normally abundant in these cells, including desmosomes as well as the E-cadherin-containing, actin filament-anchoring adhering junctions (e.g., Fig. 1 *a*), similar to what has previously been reported for various other cultured epithelial cells growing in LCM (for references see Introduction).

Double-label immunostaining with antibodies against the desmosomal plaque proteins desmoplakin and plakoglobin or against Dsg revealed punctate arrays, often with some enrichment in the juxtannuclear cytoplasm (Fig. 1 *a*, *b*, *b'*, *c*, *c'*). By double-label localization experiments with cytokeratin antibodies, sites positive for desmosomal proteins appeared in several places to be associated with and lined along IF bundles (Fig. 1, *a* and *a'*; for similar observations in other cells see references 20, 21, 37, 50, 51, 61). Again, in these IF-associated spots colocalization was observed for desmoplakin and Dsg (Fig. 1, *b* and *b'*) as well as for Dsg and the common plaque protein, plakoglobin (Fig. 1, *c* and *c'*; the additional reaction sites of plakoglobin probably reflect the occurrence of this protein in other, i.e., nondesmosomal adhering junctions; see reference 15). Colocalization of these desmosomal proteins in the same dot arrays was also observed with Dsc antibodies and in HaCaT-L and other LCM-grown cell cultures of very low cell densities (data not shown).

The observed colocalization patterns suggested that in HaCaT-L cells desmosomal proteins continuously assemble into numerous small but distinct membrane domains that are associated with a plaque structure and at certain points can be in contact with cytokeratin IF bundles.

### **Identification of Sites Reactive for Desmosomal Proteins as Half-Desmosomes**

Electron microscopy of HaCaT-L cells containing numerous dots of colocalization of desmosomal plaque proteins with Dsg and Dsc showed numerous dense plaques of varying sizes that were attached to either the plasma membrane (Fig. 2, *a* and *e–g*) or certain cytoplasmic vesicles (Fig. 2, *b–d*). Sometimes two plaque caps were seen on the same vesicle (e.g., Fig. 2 *d*,  $V_3$ ). IF bundles (Fig. 2, *a*, *b*, *h*, *i*, *bars*) were frequently attached to such plaques and sometimes appeared to connect several plaque-bearing vesicles with each other (Fig. 2 *d*). In addition, certain other vesicles resembling multivesicular bodies (MVBs) also showed an IF-associated plaque (Fig. 2, *h–j*). In their interior some of these vesicles contained only one or a few small vesicles

(Fig. 2, *h* and *j*,  $V_1$ ), whereas others were filled with many tiny vesicles (Fig. 2 *i*) or contained variously-sized dense aggregates (see Fig. 2 *j*,  $V_2$ ). Occasionally, we also observed situations indicative of an uptake of an entire plaque-bearing vesicle into an autophagic vacuole (Fig. 2 *k*).

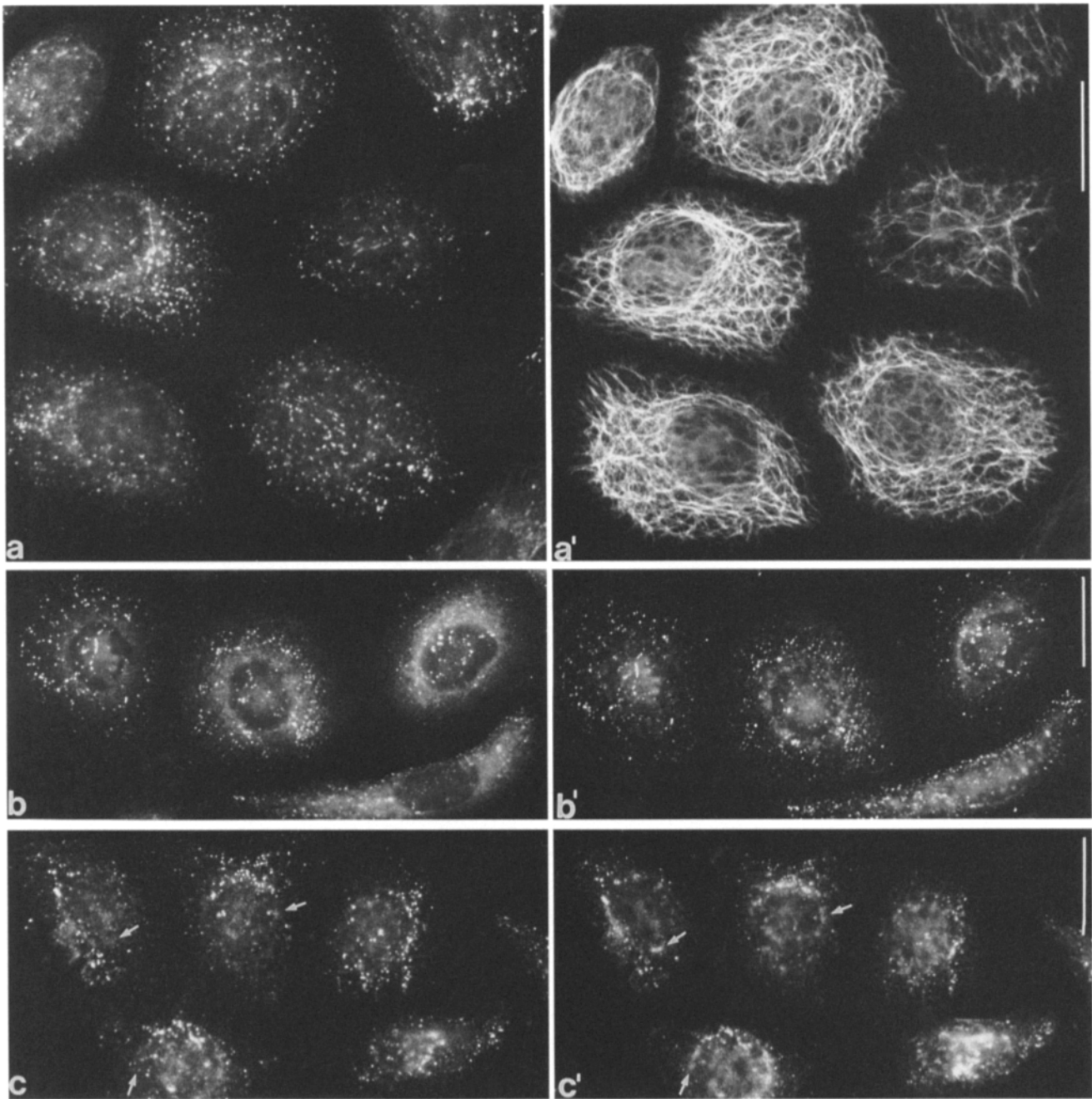
By immunoelectron microscopy single label localization of desmoplakin showed immunogold decoration of electron-dense plaques attached to these morphologically variable vesicles (Fig. 3, *a–c*). Immunogold label with Dsg and Dsc antibodies was also found at these plaque-bearing vesicles, but in general appeared more proximal to the plaque membrane (e.g., Fig. 3 *d*). This impression was confirmed by double label localization using 10-nm gold particles for desmoplakin and 5-nm gold for Dsg (Fig. 3, *e–k*) or vice versa (data not shown). We therefore conclude that in long term LCM cultures of HaCaT-L cells desmosomal proteins and glycoproteins continuously coassemble into membrane-attached plaque structures showing a half-desmosomal organization and that their formation is independent from  $\text{Ca}^{2+}$  concentrations  $>0.1$  mM and  $\text{Ca}^{2+}$ -mediated cell contacts.

### **Surface Desmoglein-Labeling of Half-Desmosomes and Their Internalization by Clathrin-Independent Endocytosis**

It is known that upon short time  $\text{Ca}^{2+}$  depletion of normal cells numerous plaque-bearing vesicles appear in the cytoplasm that result from the splitting of desmosomes and subsequent endocytosis of the resulting half-desmosomes (20, 21, 39, 40, 42, 51). To study the formation and composition of the cytoplasmic, plaque-bearing vesicles in HaCaT-L cells we used immunofluorescence microscopy with antibodies against different markers, thereby allowing vesicle typing.

Antibodies against clathrin, Dsg or Dsc both localized to frequent punctate entities distributed over the cytoplasm, sometimes showing enrichment in a juxtannuclear aggregate of dots, probably vesicles (Fig. 4, *a* and *a'*). Close comparison by double-label immunofluorescence microscopy, however, revealed that these similarly-sized, dot-like reaction sites represented two different populations of membrane structures. The spots positive for clathrin were not identical to those containing Dsg (Fig. 4, *a* and *a'*), corresponding to our electron microscopic experience of an absence of clathrin coats on the plaque-free portions of half-desmosome vesicles (see Figs. 2 and 3; references 39, 40). Similarly, comparison of the Dsg and Dsc reaction patterns with the distribution of the receptors for transferin and epidermal growth factor showed no colocalization (data not shown). This indicates that neither the clustering of desmosomal cadherins nor the endocytotic uptake of half-desmosomes follows the pathway of clathrin-mediated endocytosis of plasma membrane entities, in agreement with our and other authors' previous results (e.g., references 20, 34).

We have also compared the distribution of half-desmosomal plaque-bearing structures with lysosomal and related cytoplasmic vesicles. Immunofluorescence staining for human lysosome-associated membrane glycoprotein 1, for example, was punctate and usually concentrated near the nucleus, i.e., a region in which Dsg reaction sites often

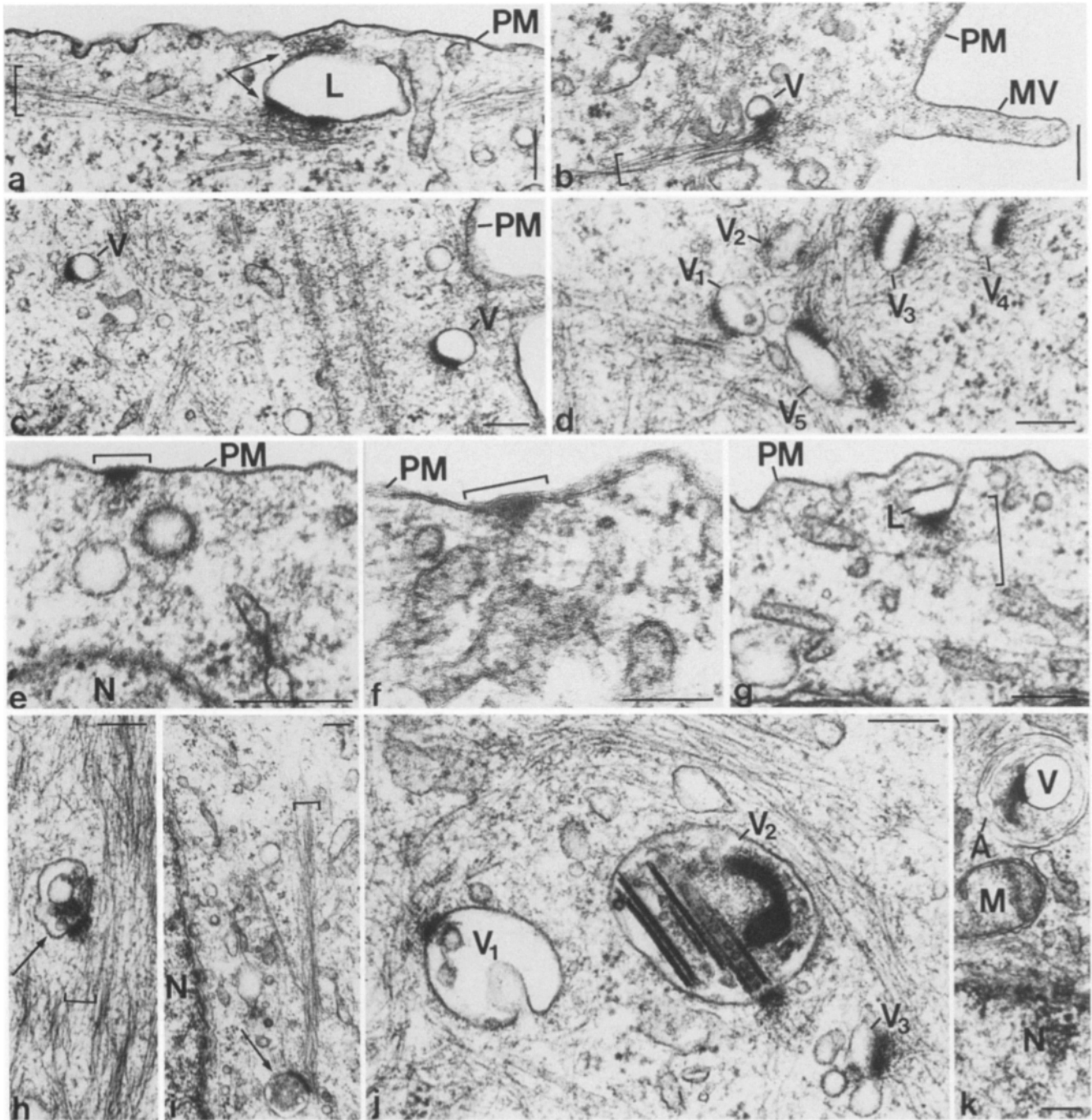


**Figure 1.** Cytoskeletal organization of human HaCaT-L keratinocytes cultured for several years in LCM as visualized by double label immunofluorescence microscopy (identical fields in *a* and *a'*, *b* and *b'*, *c* and *c'*), using antibodies for desmoplakin (*a* and *b*, from guinea pig), cytokeratins (*a'*, mAb RCK 102), desmoglein (*b'* and *c*, from rabbit), or plakoglobin (*c'*, mAb 11E4). Desmoplakin (*a*) shows the typical punctate pattern over the cytoplasm, with some alignments of fluorescent spots along cytokeratin IF bundles (*a'*). Note colocalization of desmoplakin (*b*) with desmoglein (*b'*) and of desmoglein (*c*) with plakoglobin (*c'*) in such dots. Additional diffuse immunostaining for plakoglobin (*c'*) probably reflects the association of this protein with other types of adhering junctions (see reference 15) and its soluble cytoplasmic pool (see reference 38). Arrows in *c'* denote some examples of distinct reaction sites positive for plakoglobin which are negative for desmoglein and thus also exclude cross-reaction of antibodies used. Bars, 20  $\mu$ m.

were also enriched (Fig. 4, *b* and *b'*). Detailed inspection, however, showed that the two punctate patterns were largely dissimilar. In contrast to the Dsg-containing half-desmosomes, the immunofluorescent dots of the lysosomal marker appeared larger and showed little coincidence with Dsg-reaction sites. The few occasionally noticed colabeled dots might represent individual late endosomes and

MVBs carrying a desmosomal plaque (see Fig. 2, *h-j*), assumed to be related to the lysosomal pathway (see also references 10, 30).

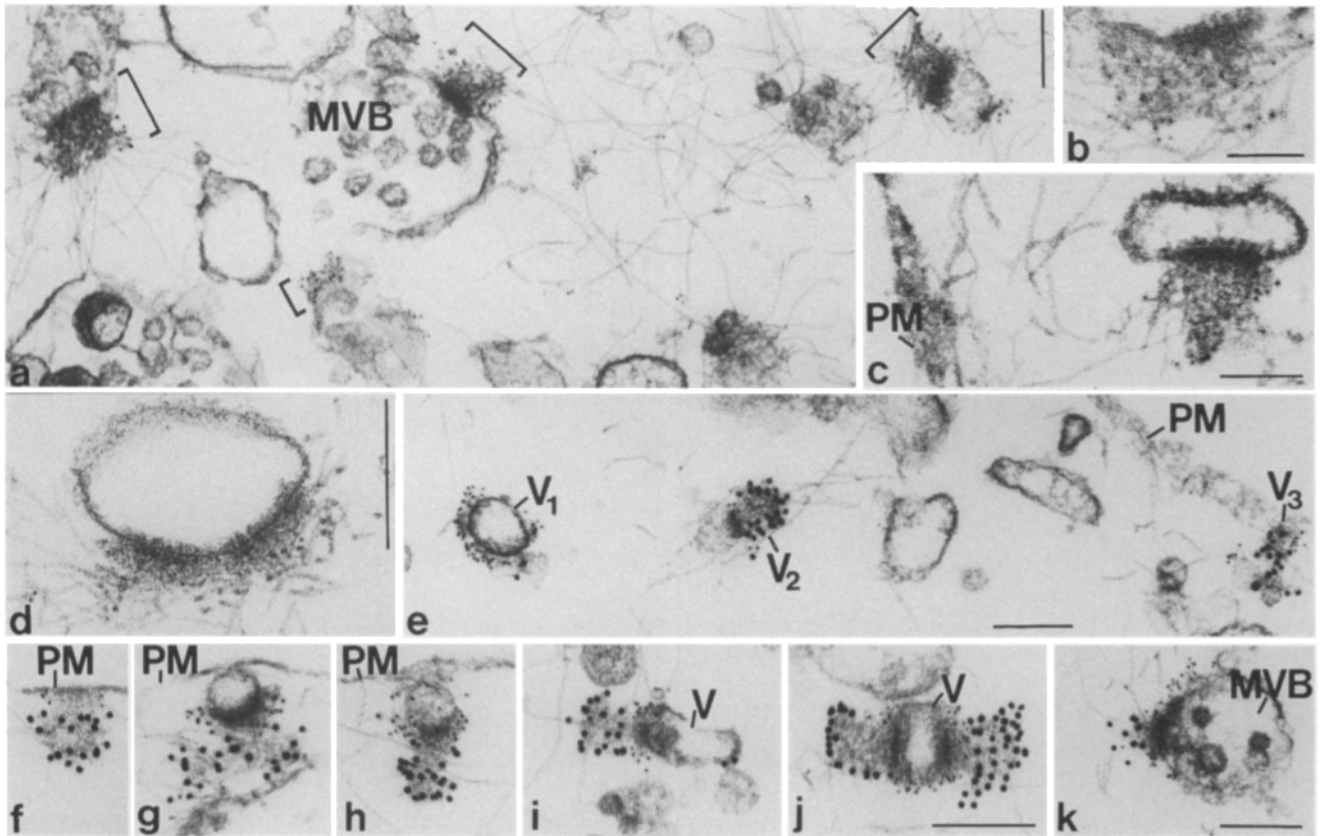
To visualize directly the endocytotic formation of the cytoplasmic plaque-bearing vesicles from internalized half-desmosomes, we used antibodies raised against extracellular parts of human Dsg2 (71) or Dsc2. On immunoflu-



**Figure 2.** Electron micrographs of ultrathin sections through keratinocytes of line HaCaT-L, showing membrane-associated desmosome-type plaque structures, frequently with anchored IF bundles. (a-d) Plaque assemblies in the cortical cytoplasm, just beneath the plasma membrane (a, PM) or somewhat deeper in the cytoplasm (b-d, MV, microvillus), are attached to vesicles bearing single (V, V<sub>1</sub>, V<sub>2</sub>, V<sub>4</sub>) or double plaque-caps (V<sub>3</sub>), which often are associated with IF bundles (a-d, brackets). (e-g) Ultrathin sections perpendicular to the plasma membrane (PM) often reveal small dense plaque structures (brackets) on the inner aspect of the surface membrane (L, lumen of vesicle or plasma membrane invagination). (h-k) Details of mostly juxtannuclear (N, nucleus) cytoplasmic vesicles resembling MVBs, which contain several small vesicles (h, i, V<sub>1</sub> in j) and/or dense aggregates (V<sub>2</sub> in j), that are associated with plaque structures and IF bundles (h, i, brackets). Sometimes autophagic vesicles (e.g., A in k) with intravesicular variously-shaped dense aggregates (j, A in k) are also observed. Bars, 0.2 μm.

orescence microscopy using intact and formaldehyde-fixed HaCaT-L cells, these antibodies showed a punctate pattern over the cell surface (e.g., Fig. 5, a and b), thus demonstrating the surface exposure of these Dsg2 epitopes in uncoupled cells.

On prolonged exposure (1–6 h) of such cells to Dsg antibodies in LCM, the number of the Dsg2-positive surface dots increased, also including some larger-appearing and more brightly fluorescent spots (Fig. 5, b–c). In most cells, dot frequencies between 30 and 120 per cell were counted.



**Figure 3.** Single (*a–d*) and double label (*e–k*) immunogold localization of desmoplakin and desmoglein at electron-dense IF-associated plaque structures at the plasma membrane or the surfaces of vesicles occurring in LCM-grown HaCaT-L keratinocytes. (*a–d*) Rabbit antibodies against desmoplakin (*a–c*) or mAb DG 3.10 against desmoglein (*d*), injected into living keratinocytes and visualized by 5-nm gold-coupled secondary antibodies, decorate electron-dense, IF-associated plaques (*brackets*; *b* shows a high magnification image) attached to cytoplasmic vesicles with a translucent lumen (*c* and *d*) and to MVBs (*a*). Note that the gold label for desmoplakin (*a–c*) is distal to the membrane and appears to be spread over the whole plaque, whereas the desmoglein label (*d*) appears closer to membrane. (*e–k*) Cytoplasmic regions beneath the plasma membrane (*PM*, *e–h*) or more distant therefrom (*i–k*) contain membrane-attached plaque structures positively labeled with rabbit antibodies against desmoplakin (10-nm gold) and mAb DG3.10 against desmoglein (5-nm gold). Note colocalization of both antigens at half-desmosomal plaques at the plasma membrane (*V*<sub>3</sub> in *e*, *f*) or at vesicle membranes (*V*<sub>1</sub> and *V*<sub>2</sub> in *e*, *g*, *h*, *V* in *i*, *j*, *MVB* in *k*). Bars: (*a*, *d–k*) 0.2 μm; (*b* and *c*) 0.1 μm.

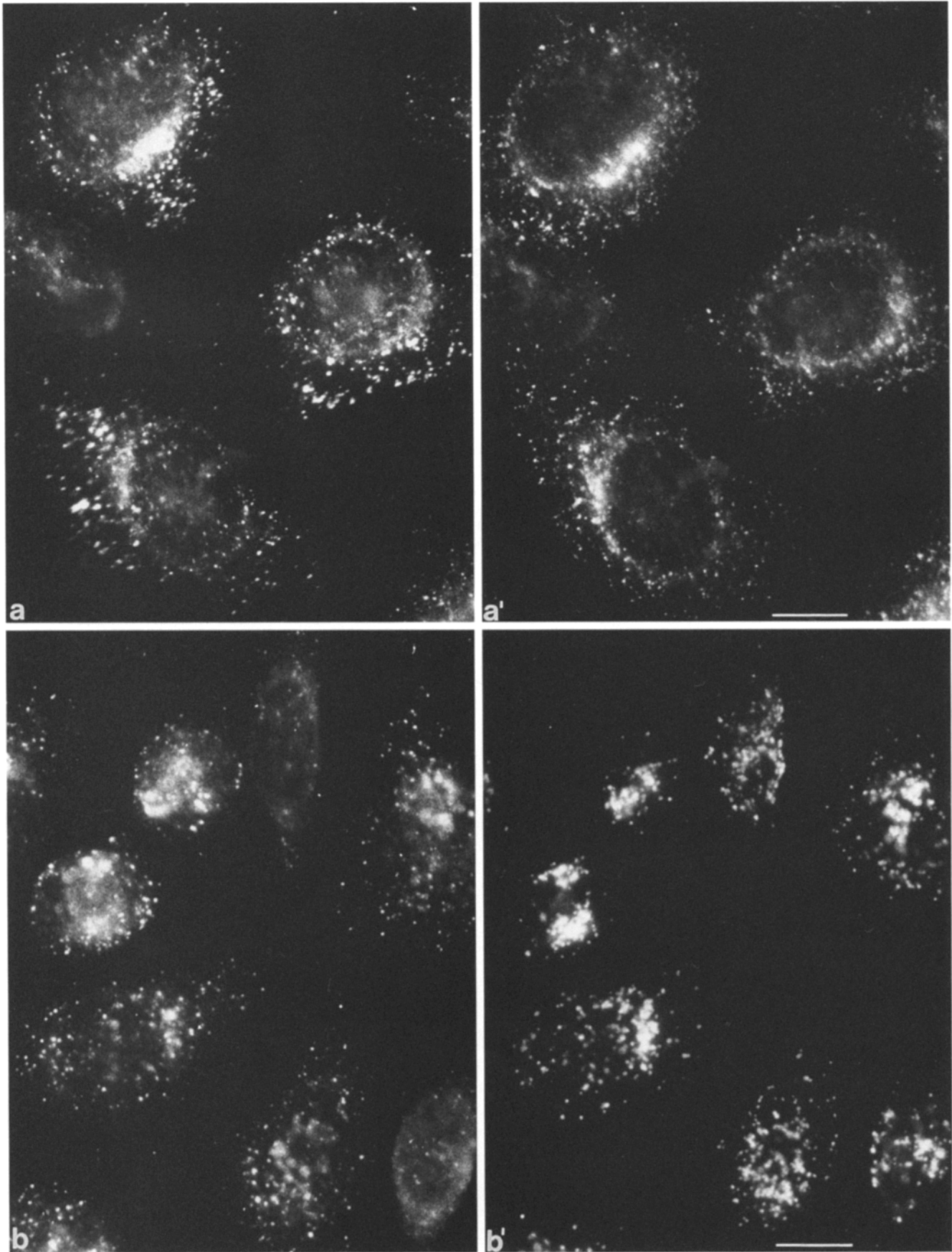
Occasionally, surface Dsg2-labeled dot- or streak-like arrays were seen locally at cell–cell boundaries and seemed to connect adjacent cells in a similar way as normal desmosomes, showing colocalization of the externally labeled Dsg2 and the internal desmoplakin (Fig. 5, *c* and *c'*). At high cell densities, such punctate contact sites positive for all four desmosomal markers tested (Dsg, Dsc, desmoplakin, plakoglobin) were often arranged in linear dot arrays resembling desmosomes (Fig. 5, *d* and *d'*).

To examine whether such surface-exposed dots positive for desmosomal markers could also be observed in NCM, i.e., in the presence of normal Ca<sup>2+</sup> concentrations allowing desmosome formation, we transferred HaCaT-L cells to NCM and plated them at low cell densities to minimize the possibilities of cell contacts. When such cells in NCM were fixed by formaldehyde and examined by immunofluorescence microscopy with antibodies to extracellularly projecting Dsg2 domains we noted a punctate pattern over large parts of the surfaces of isolated cells, including thin cytoplasmic processes (Fig. 5, *e* and *f*), which by electron microscopy were identified as variously sized, mostly very

small sites characterized by a densely stained cytoplasmic plaque, sometimes with IF bundles attached; i.e., half-desmosomes (Fig. 5, *g–j*). At higher cell densities in NCM these cells appeared to be connected by normal desmosomes whereas the numbers of half-desmosomes were greatly reduced.

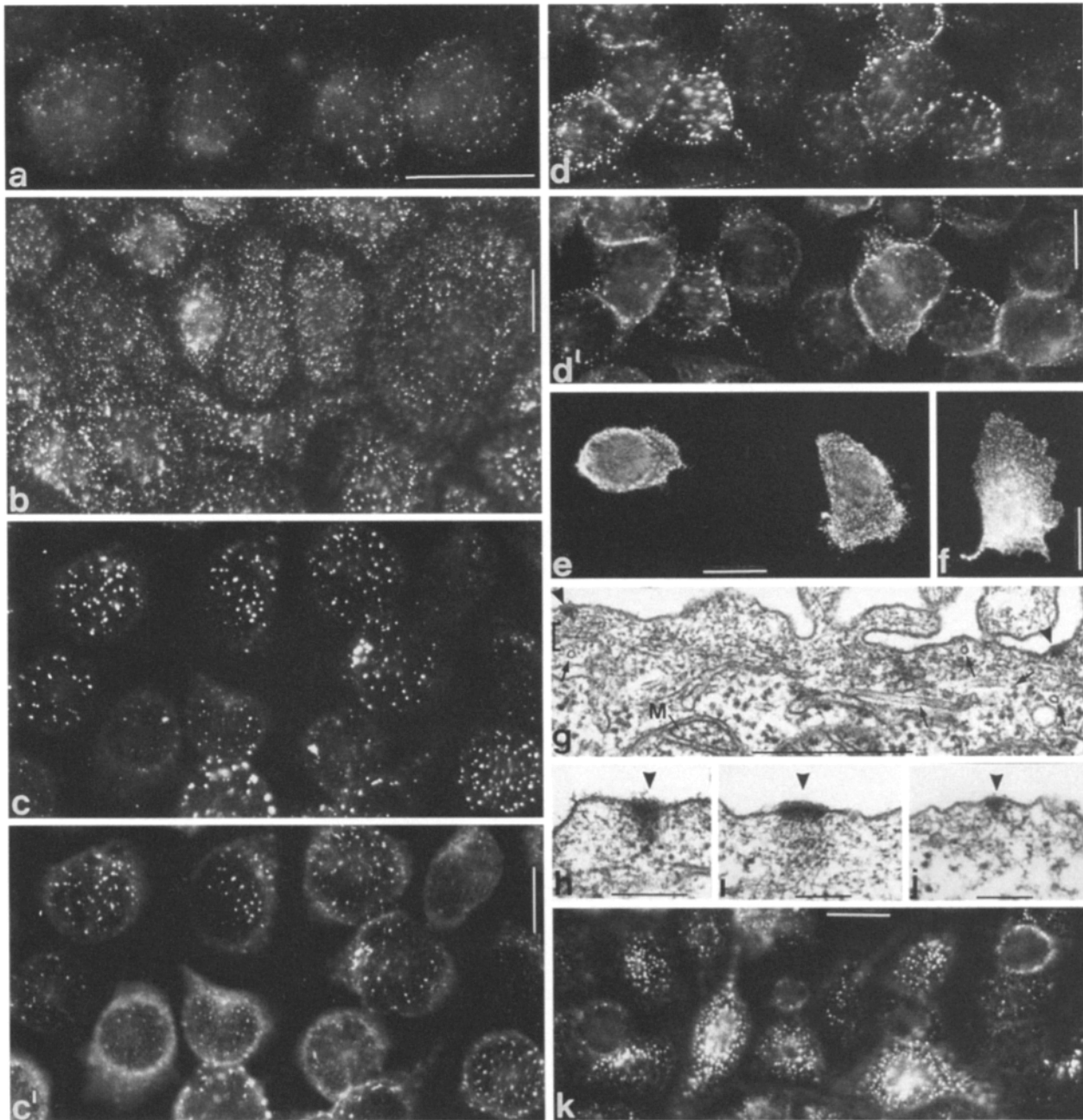
Conversely, these plasma membrane-bound half-desmosomal equivalent structures with Dsg2-domains E1–E3 projecting from the cell surface were also detected on the surfaces of cells shortly after transfer from NCM to LCM (Fig. 5 *k*). This excludes that the formation of such half-desmosomes at the surface membranes of uncoupled cells is a special property of a subclone of HaCaT-L cells selected at LCM.

We also studied the Dsg2-containing structures by immunoelectron microscopy (Fig. 6). Antibody-coupled immunogold particles added to unfixed living cells bound specifically to certain sites of the cell surface and accumulated on the outer, i.e., extracellular aspect of the half-desmosome structures (Fig. 6, *a–d*). Dsg2-specific immunogold particles were also enriched in endocytotic inpocketings

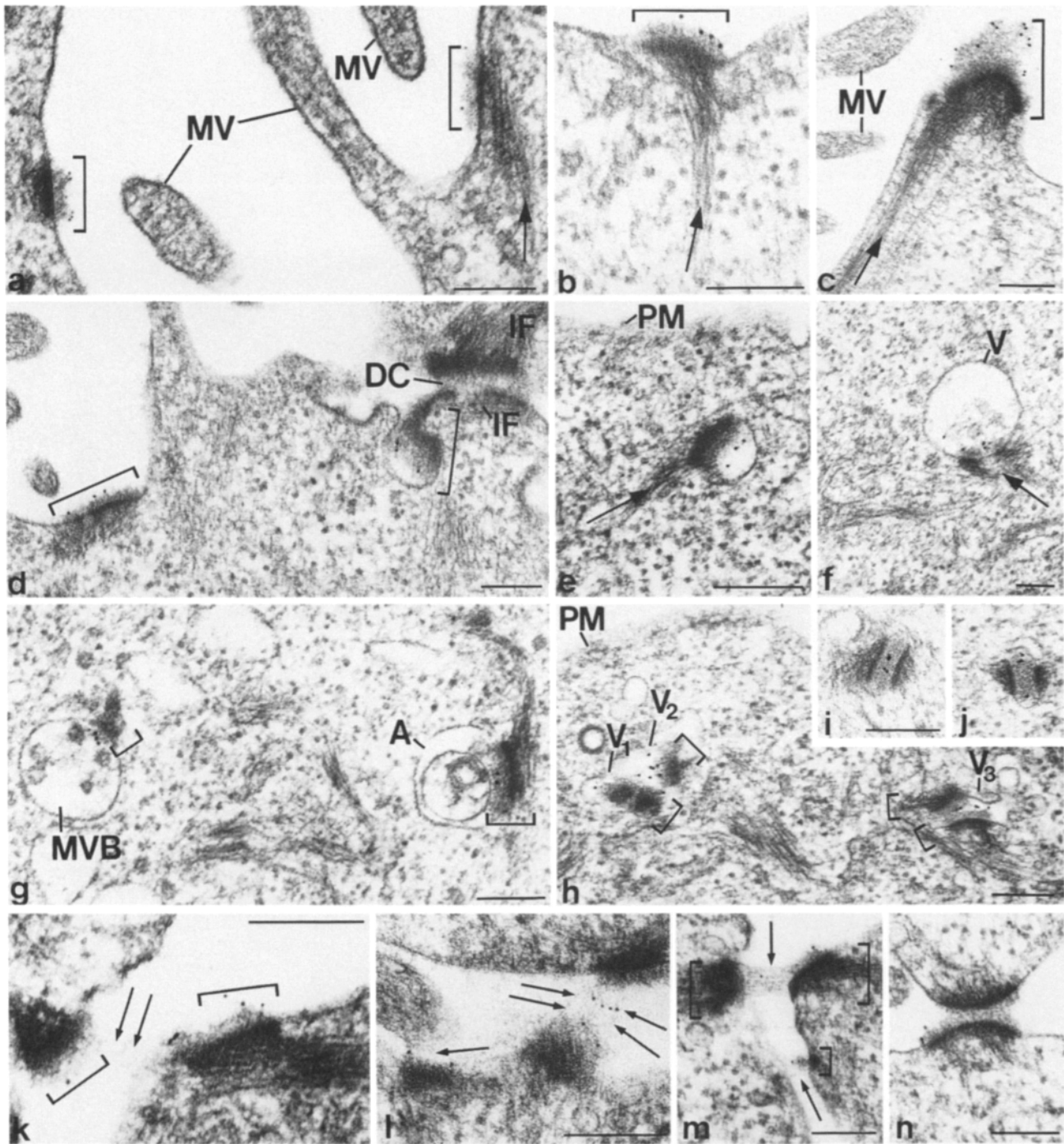


*Figure 4.* Distribution of the desmosomal cadherin, desmoglein (*a* and *b*), in comparison to clathrin (*a'*) and the human lysosome-associated glycoprotein 1 (*b'*), as revealed by double label immunofluorescence microscopy of HaCaT-L cells. Some of the fluorescent, desmoglein-positive spots (*a*, *b*, rabbit antibodies), mostly in the perinuclear cytoplasm, coincide with sites stained by antibodies against clathrin (*a'*, OD 5.9). Close examination, however, reveals that antibodies show little colocalization indicating that these desmosome half-equivalents have not been endocytosed in clathrin-coated vesicles. Antibodies against the lysosomal glycoprotein 1 (*b'*, H4A3) also reveal large spots in juxtannuclear positions, which, however, do not colocalize with desmoglein-positive half-desmosomes (*b*). Bars, 10  $\mu\text{m}$ .





**Figure 5.** Immunofluorescence (*a–e, k*) and electron (*g–j*) microscopy of HaCaT-L keratinocytes after 30 min (*a*), 60 min (*b*), or 3 h (*c–d'*) incubation of living (*a–d'*) or formaldehyde-fixed (*e–k*) cells with desmoglein antibodies (Dsg2-E2/E3) specifically reactive with an extracellular Dsg2 epitope, followed by fixation and reaction with secondary antibodies. (*a*) Upon 30-min incubation with antibodies, followed by fixation with formaldehyde and direct addition of secondary antibodies so that only surface-exposed Dsg2 molecules are detectable, Dsg2 is seen to occur in numerous small spots, probably reflecting half-desmosomes at the plasma membrane. (*b*) After longer exposure (60 min) the number of Dsg2-positive reaction sites on the cell surface has increased. (*c* and *c'*) Upon 3-h exposure to guinea pig Dsg2-antibodies followed by fixation and plasma membrane disruption with methanol/acetone treatment, and subsequent reaction with rabbit antibodies to desmoplakin, most desmoglein-positive fluorescent reaction sites (*c*) colocalize with desmoplakin. (*d* and *d'*) Similar experiment as in *c* and *c'* presenting, however, cells grown to higher cell density, shows at the cell periphery of near-confluent LCM-grown keratinocytes (HaCaT-L cells) linear arrays of spots positive for desmoglein and desmoplakin at cell–cell boundaries, illustrating antibody-mediated formation of junction-like contact structures in LCM. (*e* and *f*) Immunolocalization of desmoglein Dsg2 with antibody Dsg2-E2/E3 on cell surfaces of sparsely plated HaCaT-L cells that had been transferred from LCM to NCM for 24 h, so that no desmosomes could be formed, followed by formaldehyde fixation, before addition of antibodies. Note abundant Dsg2 reaction sites on isolated cells, illustrating the half-desmosomal equivalents over the entire cell surface (*e*), including slender cell processes (see bottom left of *f*). (*g–j*) Electron microscopy of HaCaT-L keratinocyte, transferred from LCM to NCM as shown in *e* and *f*, showing the scattered small half-desmosomes at the plasma membrane (*arrowheads*) characterized by plaques of various sized (*h–j*) some of which show bundles of IFs attached (e.g., at the bracket in *g*). Small arrows in *g* denote microtubules. *M*, mitochondrion. (*k*) Immunofluorescence microscopy (as in *e* and *f*) showing surface-exposed desmoglein Dsg2 on cells of normal NCM-HaCaT keratinocytes after transfer to LCM (for details see text). The numerous Dsg2-containing punctate reaction sites represent half-desmosomal equivalents, as also confirmed by electron microscopy. Bars: (*a–e, k*) 20  $\mu\text{m}$ ; (*g*) 0.5  $\mu\text{m}$ ; (*h–j*) 0.2  $\mu\text{m}$ .



**Figure 6.** Immunoelectron microscopy of HaCaT-L keratinocytes, incubated for 3 h with desmoglein (Dsg2) antibodies (Dsg2-E2/E3), fixed with formaldehyde and reacted with 5-nm (*a* and *d*) or 10-nm (*b*, *c*, *e*-*n*) colloidal gold-coupled secondary antibodies showing the exposure of extracellular sites of Dsg2 in half-desmosomes and on the inner surface of membranes of vesicles formed by endocytosis from such half-desmosomes. (*a*-*c*) Antibodies recognize the extracellular side of half-desmosome plasma membrane domains (*brackets*; MV, microvillus), which on their inner side are coated with a plaque anchoring cytokeratin IF bundles (*b* and *c*, *arrows*). Note fuzzy coating of the extracellular surface of these plasma membrane domains, apparently due to the immunoglobulins bound (*a*-*c*, particularly prominent in the oblique section of *c*). (*d*-*f*) Internalization of plasma membrane half-desmosomal domains covered with plaques (*d*, an example of a plaque structure appearing in the process of engulfing, near to a desmosome-like contact, DC, is denoted by the right *bracket*) leads to the formation of cytoplasmic vesicles (e.g., V, V<sub>1</sub>, V<sub>2</sub>, V<sub>3</sub>, MVB) containing endocytosed colloidal gold particles and associated with IF bundles (*arrows* in *e* and *f* and *brackets* in *g* and *h* indicate plaque structures). Note the occurrence of immunoglobulin-conjugated gold label in MVBs (*f* and *g*) or autophagic vacuoles (e.g., A in *g*), which contain plaque-bearing vesicles. In addition, sandwiche vesicles carrying on their cytoplasmic surface two distinct plaques are seen to contain immunogold label at their inner, i.e., luminal face immunogold label (V<sub>3</sub> in *h* and *i*, *j*). (*k*-*n*) Antibody-mediated bridging of plasma membrane half-desmosomes (*brackets*) of adjacent cells result in the formation of semistable junctional structures resembling desmosomes. Desmoglein-specific gold label is seen on the surface membrane (*k*, *l*, *n*) or in the bridge material (*l*, *m*) connecting the two half-desmosomal domains in a way suggestive of intercellular crosslinking (*arrows*), which finally results in the appearance of structures barely distinguishable from true desmosomes (*n*). Bars, 0.2 μm.

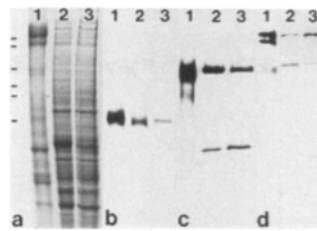
(pits) of the plasma membrane (e.g., Fig. 6 *d*), in cytoplasmic plaque-bearing vesicles of various sizes (Fig. 6, *e* and *f*), and in MVBs (e.g., Fig. 6 *g*, a grazing section through cortical cytoplasm is shown in *h*). Moreover, we consistently found externally labeled Dsg2 endocytosed in the center of peculiarly flattened desmosome-derived (tennis racket shaped) vesicles carrying a plaque on either cytoplasmic surface (Fig. 6, *i* and *j*), as they have previously been described in other cells (for review see reference 14). Such cytoplasmic vesicles were also positive for Dsc, indicating that both types of desmosomal cadherins, Dsg and Dsc, are present in the endocytosed membrane domains (for a contrasting earlier conclusion see reference 12).

In the presence of surface-bound Dsg2 antibodies such as Dsg2-E2/E3 in LCM, an increasing number of adjacent cells showed half-desmosomes in corresponding cell surface positions, with the Ig-coupled gold particles sandwiched between the outer faces of the two pairing half-desmosomes (Fig. 6, *k–m*). Final stages of these symmetrical, Dsg2 antibody-mediated desmosome-resembling junctional structures were characterized by a locally close apposition of the plaque-bearing plasma membranes, leaving only a 20–30-nm interspace filled with electron-dense intercellular material (Fig. 6 *n*). These observations suggest that half-desmosomal domains of adjacent cells are not only short-lived, unstable structures but can be cross-bridged into relatively stable junctional structures by Igs bound to the extracellular portion of Dsg2, similar to the observations made with other surface-exposed molecules (e.g., references 82, 88). Under our experimental conditions we did not notice an inhibition of cell–cell adhesion by the externally added Dsg2 antibodies as it has been reported in experiments of other authors using antibodies to various cadherins (e.g., references 3, 5, 12, 16, 29, 48, 55, 90, 92).

### Biochemical Characterization of Cytoplasmic Vesicles Containing Desmosomal Proteins

The major desmosomal proteins in HaCaT-L cells were detected by immunoblot analysis, as shown in Fig. 7 for plakoglobin (Fig. 7 *b*), Dsg (Fig. 7 *c*) and desmoplakin (Fig. 7 *d*). The gel electrophoretic positions of these proteins were identical to those of cells grown in NCM, except for lesser desmoplakin degradation (Fig. 3 *d*) and an often higher proportion of the proteolytic ~60 kD Dsg fragment comprising the cytoplasmic tail detected by mAb DG3.10 (Fig. 7 *c*, lanes 2 and 3; see references 44, 45). This somewhat higher proteolysis probably reflects the well-known increased proteolytic susceptibility of cadherins under reduced Ca<sup>2+</sup> concentration (36, 81; for review see reference 17).

To enrich the cytoplasmic structures containing these proteins we subjected a postmitochondrial supernatant from hypotonically lysed HaCaT-L cells to sucrose gradient centrifugation. Gel electrophoresis and immunoblotting of the fractions obtained (Fig. 8) revealed desmoplakin and plakoglobin of 10–16 S particles in fractions 3–5 as well as in larger particles of 40–70 S (fractions 11–16). Increasing the time of centrifugation up to 48 h did not alter the co-fractionation pattern. This indicated the existence of two forms of these cytoplasmic plaque proteins. By contrast, intact Dsg occurred in fractions 9–16, i.e., in particles sedimenting between 30 and 70 S.



**Figure 7.** Coomassie Blue-stained proteins of whole cell lysates of HaCaT keratinocytes grown under normal (lane 2) and reduced Ca<sup>2+</sup> (LCM) concentration (HaCaT-L cells, lane 3), separated by SDS-PAGE (*a*) and reacted by immunoblot (*b–d*), in comparison with desmosomal proteins from bovine muzzle epidermis (lane 1). The results show the maintained synthesis of desmosomal proteins in HaCaT cells at low Ca<sup>2+</sup> concentration. Antibodies mAb PG 5.1 against plakoglobin (*b*), mAb DG 3.10 against desmoglein (*c*) and the desmoplakin mAb mixture DP-Mix (*d*) have been used to identify desmosomal proteins in both cell culture conditions. Note an about two-fold increase of a proteolytic desmoglein fragment ( $M_r$  ~60 kD) in lysates of LCM-grown cells probably due to the enhanced proteolytic susceptibility at reduced Ca<sup>2+</sup> concentration as also known for other cadherins. Note also that HaCaT cells contain much more desmoplakin I than II (a desmoplakin mAb-reactive band at ~150 kD probably represents partially degraded desmoplakin). The most abundant proteins of isolated desmosomes from bovine muzzle epidermis are indicated at the left margin (from top to bottom: desmoplakin I, desmoplakin II, desmoglein, desmocollin I, desmocollin II, plakoglobin).

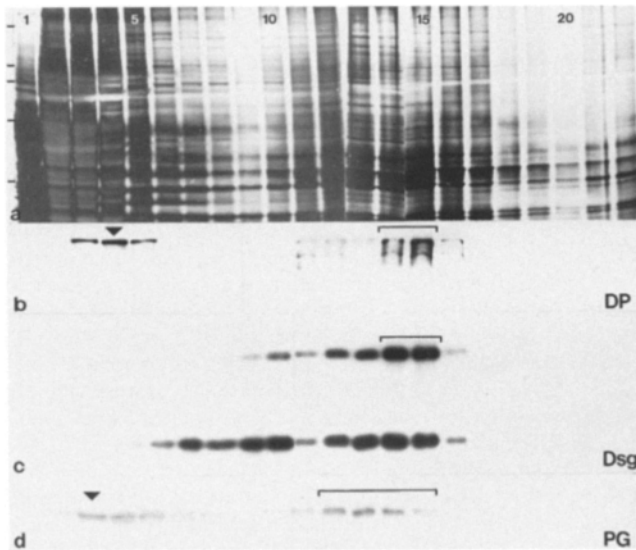
When the postmitochondrial supernatant was adjusted to 40% sucrose, overlaid with a sucrose gradient and centrifuged for 16 h, all three proteins were recovered in the same isopycnic band. These results were compatible with the interpretation of a vesicular nature of the material, indicative of a stable association of the plaque proteins to a Dsg-containing membrane. This was confirmed by immunoaffinity enrichment of the membranous structures present in pooled sucrose gradient fractions 13–16, using magnetic beads covered with Dsg mAb DG 3.4 against the cytoplasmic tail. SDS-PAGE and immunoblotting of the immunisolated structures revealed the presence of desmoplakin, Dsg, and plakoglobin (Fig. 9). Comparison of the protein pattern before and after immunoaffinity enrichment showed the specific binding of at least two further proteins of relative molecular masses 42 and 30 kD, which were not observed in control samples (data not shown).

Immunoelectron microscopy of the starting material used for affinity-purification revealed the presence of all three proteins examined in parallel (Fig. 10 *a* shows the example of Dsg) in variously sized and shaped vesicles, often with a loose filamentous coating, and (Fig. 10 *b*) plaque-like but fuzzy aggregates suggestive of residual plaque material. When the immunoaffinity-enriched, Dsg-containing structures bound to the antibody-coated Dynabeads were examined by electron microscopy, vesicles with one or two attached plaque structures were observed (Fig. 10). These findings indicated that both types of desmosomal proteins, i.e., the plaque components and the transmembrane cadherins, occurred in a special category of plaque-bearing vesicles many of which could be shown to be endocytotically derived from the IF-associated half-desmosomal assemblies at the plasma membrane.

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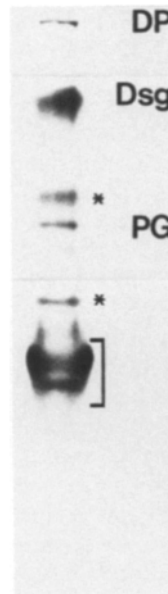
## Discussion

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**Figure 8.** Codistribution and separation of desmosomal proteins present in particles recovered in sucrose gradient fractions after velocity centrifugation of a postmitochondrial supernatant of HaCaT-L keratinocytes. (a) Silver-stained gel of gradient fractions 1–22. Molecular mass reference proteins are indicated at the left margin (from top to bottom: 200, 116, 97, 66, 45 kD). (b–d) Corresponding immunoblots in which the fractions have been probed consecutively on the same filter, with monoclonal antibodies (DP-Mix) against desmoplakin (b, DP), mAb DG 3.10 against desmoglein (c, Dsg) and mAb PG 5.1 against plakoglobin (d, PG). Note enrichment of all three desmosomal proteins in few gradient fractions, including their codistribution in fractions 12–15, as indicated by brackets (the desmoplakin cross-reactive lower band in b seems to represent a partial degradation product of desmoplakin I). The mean peak fractions of desmoplakin (fraction 14.5), of intact desmoglein (fraction 14.5) and of plakoglobin (fraction 13) are nearly identical, indicating the existence of a 40–70 S type vesicle that contains half-desmosome-equivalent assemblies. Arrowheads indicate additional peak fractions below 16 S, in which much smaller forms of desmoplakin and plakoglobin occur, probably as soluble forms (see also reference 21). The proteolytic 60-kD fragment of desmoglein shows besides its peak in fractions 12–15 a broad distribution from fractions 5–16.

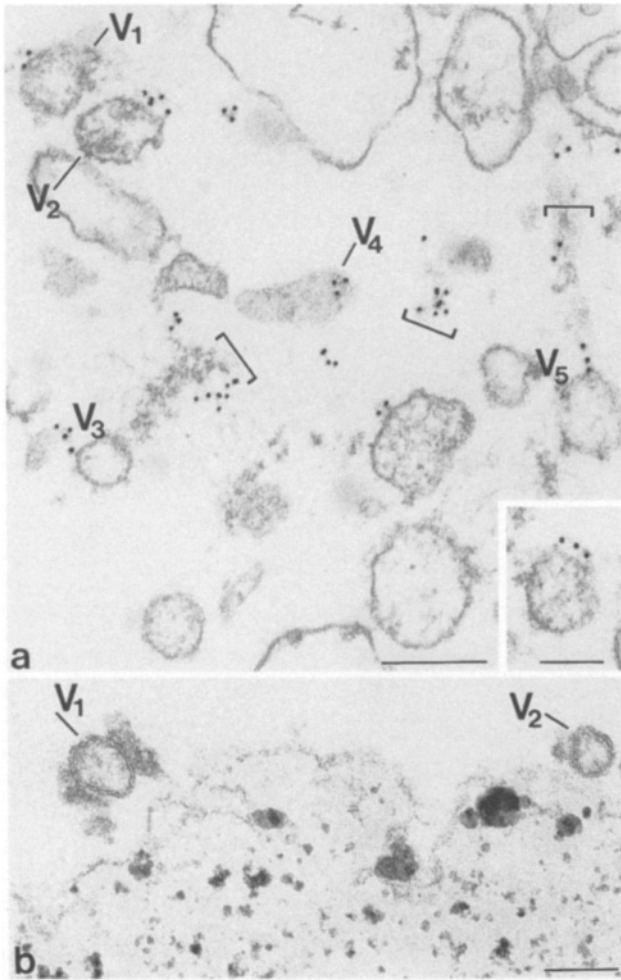
in the literature (see Introduction) unexpected result of our studies of epithelial cells growing in LCM for long periods of time (HaCaT-L cells) is that identifiable intercellular contacts are not necessary for the coordinated synthesis, processing and coassembly of desmosomal proteins and glycoproteins into plaque-bearing membrane domains with a typical desmosome-like organization and the potential to anchor IF bundles. The formation of such half-desmosomes at the plasma membrane, first noticed in human A-431 carcinoma cells grown in LCM for up to several months (21), also occurs frequently in keratinocytes (this study) and several other epithelial cell lines such as human PLC and bovine BMGE (20, 21; Demlehner, M. P., S. Schäfer, C. Grund, and W. W. Franke, unpublished results) growing in LCM and thus in the absence of cell-cell contacts: In LCM, desmoplakin, plakoglobin, Dsg, and Dsc all are not only synthesized at considerable rates but are also sufficiently stable (for reports of enhanced degradation of desmosomal molecules in LCM see references 60, 66–68) to cluster and to coassemble into numerous half-



**Figure 9.** Immunoblot of cytoplasmic, plaque-bearing vesicles enriched by immunoisolation from postmitochondrial supernatant fractions of hypotonically lysed (LCM-grown) HaCaT-L cells. Desmoplakin (DP), desmoglein (Dsg) and plakoglobin (PG) were identified as described in Fig. 8. Desmoglein-positive vesicles were enriched by affinity-absorption to magnetic beads (Dynabeads) coated with mAb DG3.4 against desmoglein and then subjected to immunoblotting with antibodies as in Fig. 8. Asterisks indicate degradation products of desmoglein and the vertical bracket demarcates the position of the heavy chains of immunoglobulins used for affinity absorption.

desmosomal structures seen at the plasma membrane and in special cytoplasmic vesicles many of which are demonstrably derived from plasma membrane-bound half-desmosomes by endocytosis (for a report of down-regulation of desmoplakin synthesis in certain bovine cells upon desmosome disruption with the tumor promoter agent, TPA, see, however, reference 4). Although we have found surface-labeled Dsg and endocytotic tracer substances in a high proportion of such vesicles (see also references 21, 39, 40, 42) we can presently not exclude that some of them may have assembled their plaques directly in the cytoplasm, i.e., without previous passage to the cell surface.

Our results have also shown that the half-desmosomal domains at the plasma membrane or at vesicles contain all major known desmosomal constituents, in contrast to previous reports concluding that in the LCM-grown HaCaT-L cells Dsg and Dsc or Dsg and desmoplakin would not colocalize (12, 60–62, 64; see also reference 37). Such half-desmosomes, i.e., variously sized plasma membrane domains with a dense cytoplasmic plaque and enriched in the characteristic desmosomal constituent molecules, occur actually much more frequently in diverse cell cultures (e.g., references 14, 21, 74, 91; see also references 18, 58) than the low number of researchers reporting them might suggest. In most previous studies in which cells were dissociated by protease and/or  $Ca^{2+}$  chelators it could, of course, not be excluded but appeared rather likely that the occasionally observed asymmetric half-desmosomal structures were residues from the splitting of preexisting desmosomes (e.g., references 18, 58, 74). Such an origin is simply not possible in the present study of long-term LCM cultures of cells devoid of any desmosome. We have no explanation why these frequent structures have escaped detection by other groups studying possible mechanisms and intermediates of  $Ca^{2+}$ -dependent desmosome formation (e.g., references 37, 51, 61, 64, 76). Perhaps, the punctate Dsc immunostaining observed on intact LCM cells by Matthey and Garrod (51) was also due to the reaction of such half-desmosomes (see also Fig. 1 of reference 87).



**Figure 10.** Electron micrographs showing particles present in sucrose gradient fractions (compare Fig. 9), obtained after centrifugation, before (a) and after (b) affinity enrichment by magnetic beads coated with mAb DG3.4 against desmoglein. (a) Desmoglein-containing vesicles ( $V_1$ – $V_5$ ;  $V_4$  is shown in a grazing section) of different sizes and shapes containing amorphous or finely vesicular material ( $V_1$ ,  $V_2$ ), have been identified by immunogold labeling using rabbit antibodies against the cytoplasmic portions of desmoglein Dsg2. Note enrichment of this gold label along the vesicular membrane (see also inset in a). Besides vesicular staining Dsg2-gold label is also found at the filamentous aggregates reminiscent of fragments of desmosomal plaques. (b) Plaque-bearing, cytoplasmic vesicles ( $V_1$ ,  $V_2$ ) are enriched after reactions of particles present in sucrose gradient fractions, obtained after centrifugation, by binding to the surface of desmoglein-antibody coated magnetic beads. Bars: (a) 0.2  $\mu\text{m}$ ; (a and b, inset) 0.1  $\mu\text{m}$ .

Our results also show that the formation of half-desmosomes is not an artefact of reduced extracellular  $\text{Ca}^{2+}$  concentrations as they are also abundant on the plasma membranes of individual, i.e., uncoupled cells growing in NCM. Therefore, we suggest that these half-desmosomes and the endocytosed vesicles derived therefrom are basic cell structures of epithelial cells in culture, frequent in isolated cells grown in the absence of the formation of symmetrical desmosomes and perhaps mostly only as intermediates of desmosome formation in coupled cells. As such structures have been occasionally also reported in diverse other cell

types and also in normal tissues and in tumors (for references see 14, 39, 40–42, 91), including human fetal epidermis (Demlehner, M. O., S. Schäfer, C. Grund, W. W. Franke, unpublished results), we think it likely that they also occur, probably as short-lived intermediates of desmosome formation, in various solid tissues. In this context it should also be mentioned that endocytosis of true hemidesmosomes connecting basal plasma membrane domains with extracellular matrix structures into IF-associated plaque-bearing vesicles has also been observed upon detachment of the cells from the matrix (59, 69; see there for further references; for possible precursors see reference 83).

Our finding that the coassembly of desmosomal cadherins with desmoplakin and plakoglobin into a plaque-bearing desmosomal equivalent structure, at the plasma membrane or in vesicles, depends neither on a  $\text{Ca}^{2+}$  concentration  $>0.1$  mM nor on cell contact formation is obviously in contrast to reports of several other groups who have concluded that cell contact formation is a prerequisite of, and actually may induce, the coassembly of these molecules at the contact site (e.g., reference 37, 60–62, 64, 68, 76). Some authors (84; see also reference 79) have further concluded that desmoplakin aggregates assemble at IF bundles deep in the cytoplasm, away from cadherin-containing membrane structures, and that these IF-attached desmoplakin plaques are direct precursors to desmosomal plaques and, on shift to NCM, rapidly translocate as IF-bound heavy weight complexes to the plasma membrane where they pair with corresponding structures of adjacent cells to form desmosomes.

While this transfer of desmoplakin aggregates upon culture medium shift to NCM has been reported to be associated with IF bundles (e.g., references 37, 61, 64, 84; see also reference 79) the transport of Dsg to the plasma membrane has been postulated specifically to involve microtubules (64; see, however, reference 63, 65). Clearly, IF bundles are not generally and absolutely needed to form and maintain desmosomes as perhaps best indicated by the desmosomes of IF-lacking murine embryonal cells (2) and the toxically deranged mouse hepatocytes in which all detectable cytokeratin material is aggregated into so-called Mallory bodies usually away from the desmosomes (19). Our impression, in line with previous observations in our laboratory (20, 21) and elsewhere (51), is different: in LCM, both kinds of partners, the desmosomal cadherins Dsg and Dsc and the cytoplasmic plaque proteins, are continually coassembled into half-desmosome structures that under these conditions are, however, not stabilized by the last  $\text{Ca}^{2+}$ -dependent step, i.e., formation of desmosomes with a corresponding domain of an adjacent cell, but are frequently resumed in a frustrated cycle, into the cytoplasm by a special pathway of endocytosis.

The results of the present study also leave no doubt that at least some regions of the extracellular repeating domains of Dsg glycoproteins are exposed on the cell surface and readily accessible to Igs (for related observations in complete desmosomes of tissues see also 80). The demonstrated surface exposure of parts of Dsg2 also corresponds to the concept that pathogenic auto-antibodies present in patients suffering from the blistering diseases of the skin and other stratified squamous epithelia, *Pemphigus folia-*

ceus (against Dsg1) and *Pemphigus vulgaris* (against Dsg3), bind to certain parts of extracellular Dsg domains (for review see reference 78).

Our finding that antibodies directed to an extracellular domain of Dsg2, when added to living cells, bind to the cell surfaces of uncoupled cells should be seen together with the results of Cowin et al. (12). These authors have emphasized that their Dsg antibodies and Fab' fragments prepared therefrom did not at all bind to the cell surface of living or fixed intact epithelial cells, in contrast to Dsc antibodies applied in parallel. To us the simplest and most plausible explanation for this negative result, also mentioned inter alia by these authors, is that their Dsg antibodies used did not react with an epitope in the extracellularly exposed part of the specific Dsg isoform(s) present in the cells examined but reacted with other Dsg segments, probably the cytoplasmic tail domain, corresponding to our results with Dsg-mAb 3.10 (44–46, 72, 73).

Obviously, our finding that half-desmosomes are frequent cell surface structures in the absence or reduction of desmosome formation also opens the possibility to identify, sort and treat cell states transiently lacking, or poor in, desmosomal cell contacts such as embryonal cells and carcinoma metastatic cells of certain stages with antibodies to Dsgs or Dscs or other specific ligands.

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