

Identification of an Epithelial Protein Related to the Desmosome and Intermediate Filament Network

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Abstract. Using a mAb, referred to as 08L, we have identified a protein, of $M_r \approx 140,000$, associated with desmosomes of epithelial cells. The 08L antibody stained the intracellular side of lateral cell margins of monolayer epithelial cells but did not stain cell margins free of cell contact. Immunoelectron microscopy revealed that the 08L antigen was localized to the cytosolic surface of the desmosomal plaque near points of intermediate filament convergence with apparently little staining of the desmosomal plaque proper. Western blots revealed the 08L antigen to be a protein, of $M_r \approx 140,000$, found in the Triton-X 100 insoluble pellet. High salt-containing buffers extracted the 08L antigen from the insoluble material. Examination of the as-

sembly of 08L to the desmosome complex, in cells grown in low confluent culture or in calcium-switch assays, by double immunofluorescence with 08L and anti-desmoplakin antibody, revealed that 08L was recruited to morphologically identifiable desmosomes. 08L antigen may exist in a cytosolic pool prior to assembly to the cell surface. The solubility of 08L in low calcium and normal calcium conditions, however, was similar. 08L association to the desmosome was correlated with increased organization of the intermediate filament network. We suggest that the 08L antigen may be involved in the organization and stabilization of the desmosome-IF complexes of epithelia.

THE desmosome (macula adherens) is a major component of the epithelial intercellular junctional complex. Desmosomes are intimately involved in the structural and functional integration of adjacent epithelial cells (Fey et al., 1984; Cowin et al., 1985a; Garrod et al., 1990). The desmosome serves as a site of reinforcement of cell-cell adhesion as well as an anchorage point for the intermediate filament scaffold of the cell. Therefore, the desmosome is integral in epithelial cell and epithelial sheet organization. The intercellular space at the desmosome is 20–30 nm and contains an electron-dense midplate. A pair of dense cytoplasmic plaques are found beneath lateral cell membranes and are associated with looping bundles of intermediate filaments radiating from the cytoplasm.

Because of the availability of isolation procedures for desmosomes, detailed morphological, biochemical, and molecular analyses of desmosomal components have led to the identification of, and putative function for as many as eight desmosomal proteins (Drochmans et al., 1978; Suhrbier and Garrod, 1986; Schwartz et al., 1990). The constituent proteins include desmoplakins (I and II) which are non-glycosylated proteins ($M_r \approx 280,000$ and $250,000$, respectively) found within the desmosomal plaque (Mueller and Franke, 1983; Green et al., 1990), plakoglobin ($M_r \approx 83,000$), a plaque protein which is apparently related to the recently described catenins (Gorbsky et al., 1985; Cowin et al., 1986; Franke et al., 1989; McCrea et al., 1991), desmocollin (I and II) ($M_r \approx 115$ – $130,000$), and desmoglein

($M_r \approx 160,000$) which are Ca^{2+} sensitive cell adhesion transmembrane glycoproteins with distinct homology to the cadherins (Cowin et al., 1984; Schmelz et al., 1986a,b; Holton et al., 1990; Koch et al., 1990; Collins et al., 1991; Magee and Buxton, 1991; Mechanic et al., 1991). Other desmosomal components have been reported to be found in a limited subset of desmosomes, such as desmoplakin IV or band 6 polypeptide (Kapprell et al., 1988). In addition, still other reports of minor or cell specific desmosomal-associated proteins are plentiful (Tsukita and Tsukita, 1985; Jones et al., 1986; Jones, 1988; Hieda et al., 1989). The molecular complexity of the desmosome may be indicative of its functional diversity and tissue heterogeneity. Although much is known about desmosome composition, many questions remain concerning the specific role of individual components of the desmosomal complex, their regulation, and means of assembly and disassembly.

As mentioned above, the desmosome serves as an anchorage point for intermediate filaments (IF)¹. However, the specific molecular entities involved in IF binding at the desmosome remain uncertain. Location on the cytoplasmic portion of the desmosomal plaque (Jones and Green, 1991) and the predicted molecular structure of desmoplakin iden-

1. *Abbreviations used in this paper:* CSK, cytoskeleton; IF, intermediate filament; IgM, immunoglobulin-M; LCM, low calcium medium; NCM, normal calcium medium.

tify it as a putative IF linker (Green et al., 1990), yet, no firm biochemical evidence exists for direct IF-desmoplakin interaction (O'Keefe et al., 1989). Other desmosomal proteins such as desmocalmin and desmoplakin IV have been shown to bind IFs in vitro (Tsukita and Tsukita, 1985; Kaprell et al., 1988), however, these proteins exhibit a limited tissue distribution and, therefore, would be unlikely candidates for general mediators of IF-desmosomal interactions. Recently, Cartaud et al. (1990) have reported a desmosomal plaque protein with $M_r \approx 140,000$ which is related to lamin B, the IF receptor of the nuclear lamina. This protein has been shown to bind vimentin filaments.

Here, we report the identification of a desmosome-IF complex-associated protein, $M_r \approx 140,000$, which is associated with most if not all desmosomes. This protein was localized to the periphery of the desmosomal plaque and adjacent amorphous material near the convergence of intermediate filaments. This 140-kD protein assembles to preformed desmosomes, and therefore, is not integral to desmosomal structure. However, the presence of 08L at the desmosome is correlated with the organization of keratin filament network. We speculate that this protein may be relevant to desmosomal stability and involved in other functions of the desmosome such as intermediate filament organization and assembly.

Materials and Methods

Reagents

DME and suspension-MEM (S-MEM) were purchased from Gibco-BRL (Grand Island, NY). RPMI medium, Hanks medium, and FCS, for growing hybridoma cells were purchased from Irvine Scientific (Santa Ana, CA). FCS, used for culture of MDCK cells, was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). PMSF, leupeptin, chemostatin, and pepstatin were purchased from Sigma Chemical Co. (St. Louis, MO). Unconjugated and FITC-conjugated goat anti-mouse IgM were purchased from Chemicon International, Inc. (Temecula, CA). mAb against pan-cytokeratin, FITC-conjugated goat anti-mouse IgG, and IgM and peroxidase-conjugated goat anti-mouse IgM were purchased from Boehringer-Mannheim Corp. (Indianapolis, IN). Texas red-conjugated goat anti-rabbit IgG was purchased from Cappel (Organon Teknika Corp., Durham, NC). Type I collagen was purified from rat tail tendons as described by Dodson and Hay (1971). Rabbit polyclonal anti-desmoplakin antibody was kindly provided by Dr. Manijeh Pasdar (University of Alberta in Edmonton, Canada).

mAb Production

MDCK cells grown in 150-mm² tissue culture flask were harvested with a rubber policeman and lysed in 20 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA containing 1% NP-40 for 60 min at 4°C. Detergent soluble fractions were used as immunogen and mixed with complete Freund's adjuvant to inject Balb/c mice. Subsequent to three or four boost injections, at 2-wk intervals, mice were sacrificed and splenocytes fused with P3 myeloma cells, similar to that described by Köhler and Milstein (1975). Hybridoma cells grown in HAT-containing RPMI medium were plated into 24-well dishes. Supernatants were screened by immunofluorescence microscopy of MDCK cells cultured on collagen gels and dot-blot using whole cell protein extracts. Cells of positive wells were selected and cloned by limited dilution. We thus obtained a mAb, designated 08L, which was an IgM. For Western blot, 08L-IgM was concentrated with 40% ammonium sulfate followed by extensive washing with 25 and 20% ammonium sulfate.

Purification of IgM

Goat anti-mouse IgM was coupled to CNBr activated Sepharose-4B according to manufacturer's instructions (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). The anti-immunoglobulin-M (anti-IgM) Sepharose affinity column was pre-equilibrated with 20 mM Tris, pH 8.0 (running buffer), and washed sequentially with 0.1 M glycine, pH 2.6 (elution buffer),

20 mM Tris, pH 8.8, 0.1 M triethylamine, pH 11, and running buffer. 08L hybridoma supernatant was filtered through a 0.2- μ m filter before passing to an anti-IgM column. The bound IgM was eluted from the column with elution buffer, neutralized with 1 M Tris, pH 8.0, dialyzed extensively against PBS, and stored at -20°C.

Cell Culture

Cell cultures of the MDCK cell line of passage 10-60 (provided by Dr. Karl Matlin, Harvard Medical School, Boston, MA), were maintained in DME supplemented with 10% FCS, 2 mM glutamine, and 200 U/ml each of streptomycin and penicillin G (normal calcium medium, NCM). Cells were passed with 0.1% trypsin and 0.04% EDTA in Hanks medium. For MDCK cells cultured on collagen gel, a Lab-Tek culture chamber (Nunc Inc., Naperville, IL) precoated with type I collagen was used. For calcium signaling assays, confluent MDCK cells grown on coverslips in NCM were changed to low calcium medium (LCM) for 2 d and then shifted back to NCM for varied time points before immunostaining (see below). LCM had the same formulation as NCM with the exceptions that calcium salts were omitted by replacing DME with S-MEM and the FCS was depleted of divalent cation by using Chelex membrane (Bio-Rad, Richmond, CA).

Immunofluorescence Microscopy

MDCK cells grown to confluence on collagen gels were scraped from the culture chamber with a razor blade. Samples were frozen and mounted in Tissue Tek[®] OCT embedding medium (Miles Scientific, Naperville, IL) and 8- μ m sections were cut on a cryostat. Cells on coverslips were permeabilized with acetone for 3 min at -20°C. Sectioned material and coverslips were washed briefly in PBS and incubated with 08L supernatant for 1 h at room temperature. After three 5-min washes in PBS, the samples were incubated with FITC-conjugated goat anti-mouse IgM diluted 1:100 in PBS for 1 h at room temperature. The samples were then washed extensively in PBS, mounted with 50% glycerol containing 0.4% *n*-propylgallate and examined with a photo microscope (Carl Zeiss, Oberkochen, Germany) equipped with epifluorescence. Double immunofluorescence microscopy used primary antibody mixtures of 08L supernatant/anti-desmoplakin (diluted 1:200), 08L/anti-cytokeratin (diluted 1:10) and anti-desmoplakin/anti-cytokeratin.

Immunoelectron Microscopy

Purified 08L IgM was dialyzed extensively against 20 mM sodium borate, pH 9.0, and coupled to 10 nm colloidal gold as described by Larsson (1988). 08L-conjugated colloidal gold was concentrated by centrifugation at 45,000 g for 1 h using a rotor (SW60; Beckman Instruments, Inc., Palo Alto, CA) and resuspended in 20 mM Tris, pH 7.6, containing 150 mM NaCl, 1% BSA, and 0.02% sodium azide. For immunogold labeling, 17-d-old embryonic chick corneal epithelia were detached from underlying stroma with 0.4 mg/ml EDTA, permeabilized with buffer containing 0.5% Triton-X 100 for 30 min at 4°C, and incubated with 08L-conjugated colloidal gold for 1 h at 4°C. Alternatively, MDCK cells cultured on coverslips were fixed briefly in 1.5% formaldehyde, washed with PBS, permeabilized, and finally incubated with 08L-gold. Subsequent to the 08L-gold incubation, the epithelia and MDCK cells were washed extensively with 20 mM Tris, 150 mM NaCl containing 1% BSA and fixed with 2.5% glutaraldehyde, 2% formaldehyde plus 1% picric acid buffered in 0.1 M cacodylate for 1 h. After washing in 0.1 M cacodylate buffer, the samples were postfixed in 1% osmium tetroxide, en bloc stained with uranyl acetate, and prepared by routine methods for EM. Thin sections were viewed on an electron microscope (1200; JEOL USA, Peabody, MA).

Protein Extracts

MDCK cells in culture dishes were scraped with a rubber policeman and homogenized briefly in either cytoskeleton (CSK) buffer (Fey et al., 1984; 10 mM Pipes, 300 mM sucrose, 150 mM NaCl, 3 mM MgCl₂, 1 mM EDTA, 0.1 mM DTT and 0.5% Triton-X 100, pH 6.8, 1 mM PMSF, and 1 μ g/ml each of pepstatin, leupeptin, and chemostatin) or CSK buffer containing 1.5 M KCl. Cells were lysed on ice for 30 min followed by centrifugation for 30 min at 10,000 g at 4°C. The supernatants and pellets were subjected to SDS-PAGE and Western blotting.

SDS-PAGE and Immunoblot

Protein extracts as described above were resuspended in sample buffer and

electrophoresed on 6% polyacrylamide gels according to the system of Laemmli (1970). Immunoblot analysis was performed as described by Towbin et al. (1979) with a few modifications. Proteins were transferred from the gel to nitrocellulose paper (Schleicher & Schuell, Keene, NH). The paper was blocked with 5% non-fat dried milk in PBS followed by washing in PBS. Primary antibody incubations were carried out with 08L concentrated by ammonium sulfate for 1 h at room temperature. After extensive washing in PBS, the paper was incubated for 1 h at room temperature with HRP conjugated goat anti-mouse IgM (1:1000 in PBS). The peroxidase-labeled blots were reacted with 0.5 mg/ml diaminobenzidine in PBS and color reactions were developed using 0.01% hydrogen peroxide.

Differential KCl Extraction

A series of confluent MDCK cells, cultured in 60-mm cultured dishes, were extracted *in situ* with CSK buffer containing different concentrations of KCl (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.5 M) at 4°C for 30 min. After KCl extraction, cellular remnants remaining in the culture dishes were boiled in sample buffer and collected. Both the KCl extracts and salt-insoluble material were subjected to SDS-PAGE and immunoblot as described above. In parallel, MDCK cells, cultured on coverslips coated with either polyornithine or ConA, were extracted with KCl, as above, and processed for immunofluorescence with 08L.

Results

The mAb, 08L, which was derived from ongoing studies on epithelial adhesion, presented an intriguing lateral epithelial boundary staining pattern. Immunofluorescence microscopy of permeabilized monolayer cultures of densely grown MDCK cells revealed the distribution of 08L antigen along cell boundaries corresponding to cell-cell contact regions (Fig. 1 *a*). The epitope appeared to reside on or near the cytoplasmic face of the lateral membrane. Free cell boundaries along the margins of epithelial colonies exhibited no staining. No staining of nuclear envelope, Golgi complexes, or other distinct cytoplasmic structures was seen (Fig. 1, *a* and *b*). Sections of MDCK cells grown on collagen gels demonstrated the 08L antigen to be localized to the lateral borders of the cells with no apical or basal staining (Fig. 1 *b*).

The mAb, 08L, stained stratified epithelium as well as simple epithelium of numerous species. Immunostaining of rat stomach revealed that 08L was primarily localized to the lateral surface of epithelial cells, especially evident at lateral cell boundaries near the junctional region (Fig. 1 *c*). Sections of rat liver (Fig. 1 *d*) stained with 08L demonstrated staining along the lateral plasmalemma of hepatocytes and presented a pattern parallel to the course of bile canaliculi. Strong immunofluorescence was also observed along epithelial lateral boundaries in the bile duct (Fig. 1 *d*). Immunofluorescence of rat corneal epithelium clearly demonstrated 08L antigen at the lateral cell boundaries of basal cells and lateral surfaces of the stratifying wing cells (Fig. 1 *e*). Epidermis stained with 08L demonstrated staining of the lateral surface of all cell layers except the stratum corneum (Fig. 1 *f*). Interestingly, 08L did not immunostain the basal epithelial surfaces of corneal epithelium or epidermal cells, both of which contained well formed hemidesmosomes. In all tissues surveyed, we found no 08L staining in connective tissues, such as lamina propria of the gut, corneal stroma, or dermis. In addition, endothelium, smooth muscle, and nervous tissue appeared negative for 08L staining.

Immunoelectron microscopy revealed the 08L antigen to be associated with the cytosolic face of desmosomes. Chick corneal epithelia incubated with colloidal gold-conjugated 08L subsequent to detergent permeabilization (Fig. 2, *a*, *b*,

and *d*) and MDCK cells which were fixed and then detergent permeabilized and incubated in 08L-gold (Fig. 2 *c*) exhibited similar immunostaining patterns. The 08L antigen was localized primarily to the periphery of desmosomes and to intermediate filaments immediately adjacent to the desmosome (Fig. 2). Gold particles did not appear to be associated with desmosome plaque proper, even in areas where the plaque appeared clearly accessible, rather they were associated with amorphous material adjacent to the plaque and with IFs in the immediate vicinity of desmosomes. Samples, fixed before immunogold labeling, demonstrated a better preserved cytoarchitecture and a more dense IF meshwork. The gold labeling was still restricted to the areas immediately adjacent to the desmosome, indicating that the immunolocations in the extensively extracted epithelia did not result from a collapsing of 08L onto the desmosome. This EM distribution of 08L antigen suggests that 08L may recognize a novel component of the IF-desmosome complex.

The 08L antibody, when used to immunostain Western blots of extracts from confluent MDCK cells, revealed a prominent immunoreactive band with a relative molecular mass of 140,000 (Fig. 3, lanes 9-11). The 08L antigen displayed the same mobility on SDS-PAGE under reducing and non-reducing conditions. This 140-kD protein was not present in the Triton-X 100 soluble fraction from MDCK cells (Fig. 3, lane 8), but was observed in the detergent-resistant fraction (Fig. 3, lane 9). However, the 140-kD detergent-resistant protein could be extracted from the CSK-insoluble pellet (Fig. 3, lane 10) or directly from lysed cells (Fig. 3, lane 11) with buffers containing KCl (Fig. 3, lane 10 and 11), leaving a small residual amount of 08L protein in the KCl insoluble pellet (Fig. 3, lane 12).

The solubility of 08L antigen in KCl was examined in more detail by differential salt extraction combined with immunoblot and immunofluorescence analyses. 08L antigen was soluble at concentrations greater than 0.4 M KCl (Fig. 4 *A*). A small, but significant, portion remained insoluble even after 1.5 M KCl extraction. The immunofluorescence analyses supported the immunoblot profile with the diminishing of immunostaining with 08L following 0.4 M KCl extraction and the appearance of weak residual staining on the MDCK lateral cell surfaces even subsequent to 1.5 M KCl extraction (Fig. 4 *B*). MDCK cell cultures extracted with KCl also revealed weaker 160-170-kD immunoreactive bands. At this time, it is not clear how these larger polypeptides are related to the 140-kD 08L polypeptide.

Under normal physiological conditions, 08L antigen was seen to co-localize with desmoplakin on densely grown MDCK cells. Immunostaining of low confluent cultures of MDCK cells with 08L and anti-desmoplakin suggested that 08L assembled onto the lateral margins subsequent to desmoplakin. Immunostaining of single cells demonstrated 08L and desmoplakin to be intracellular with little or no co-distribution (Fig. 5, *a* and *a'*). Desmoplakin staining was concentrated to the paranuclear area. Staining of small cell clusters revealed that desmoplakin assembled to the cell-cell contact surfaces before 08L (Fig. 5, *c* and *c'*). The larger clusters exhibited extensive 08L-desmoplakin co-alignment, similar to that seen on confluent cultures (Fig. 5, *d* and *d'*).

Taking advantage of the observation that the structural integrity of desmosomes is affected by extracellular calcium (Hennings et al., 1980), we cultured MDCK cells at high density in low calcium medium (LCM) for 2 d. Subse-

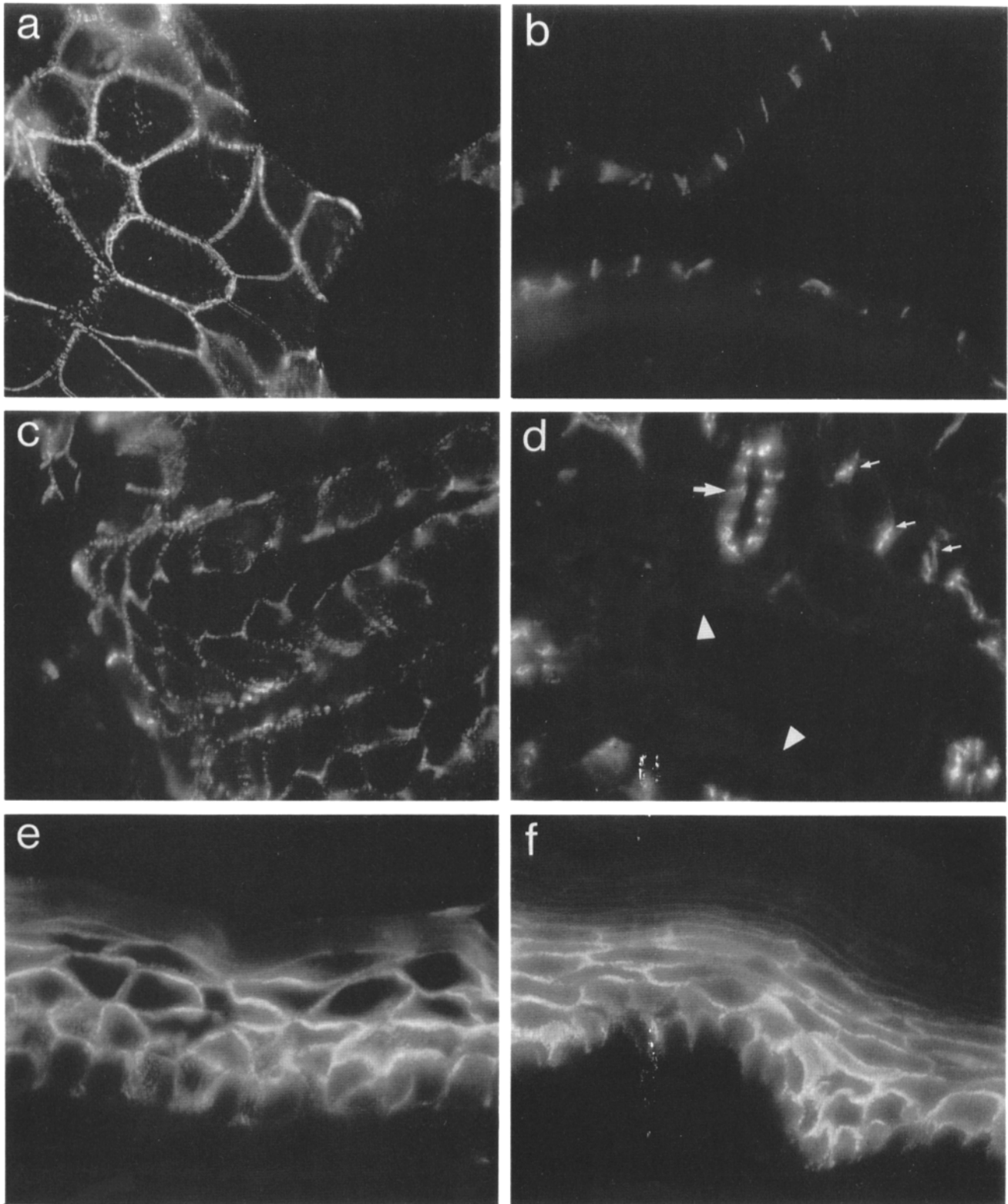


Figure 1. Confluent culture of MDCK cells stained with O8L, using routine indirect immunostaining procedures (a) revealed strong staining for O8L along the cell boundaries, corresponding to cell-cell contact sites, with a characteristic pattern of double parallel interrupted lines. Note the apparent negative staining of O8L at cell margins with no cell-cell contact. MDCK cells cultured on collagen grown to confluence, cut perpendicular to the plane of substratum (b) showed O8L staining restricted to a linear pattern at the lateral cell boundary. Little or no staining was seen intracellularly or associated with apical or basal surfaces. Sectioned rat stomach (c) showed that O8L antigen was localized to the lateral surface of epithelium, while the cells of connective tissues displayed little or no reactivity. Rat liver stained with O8L (d) demonstrated staining along the lateral surface of hepatocytes and presented a pattern representative of the course of bile canaliculi (small arrows). Bile duct epithelium also stained with O8L antibody (large arrow), whereas, the hepatic endothelium did not stain (triangles). Rat cornea (e) and rabbit skin (f) showed epithelial staining for O8L, however, underlying connective tissue is not stained. Note lateral epithelial surfaces stained with O8L whereas the basal surfaces of basal epithelial cells which contain numerous hemidesmosomes showed no immunoreactivity for O8L.

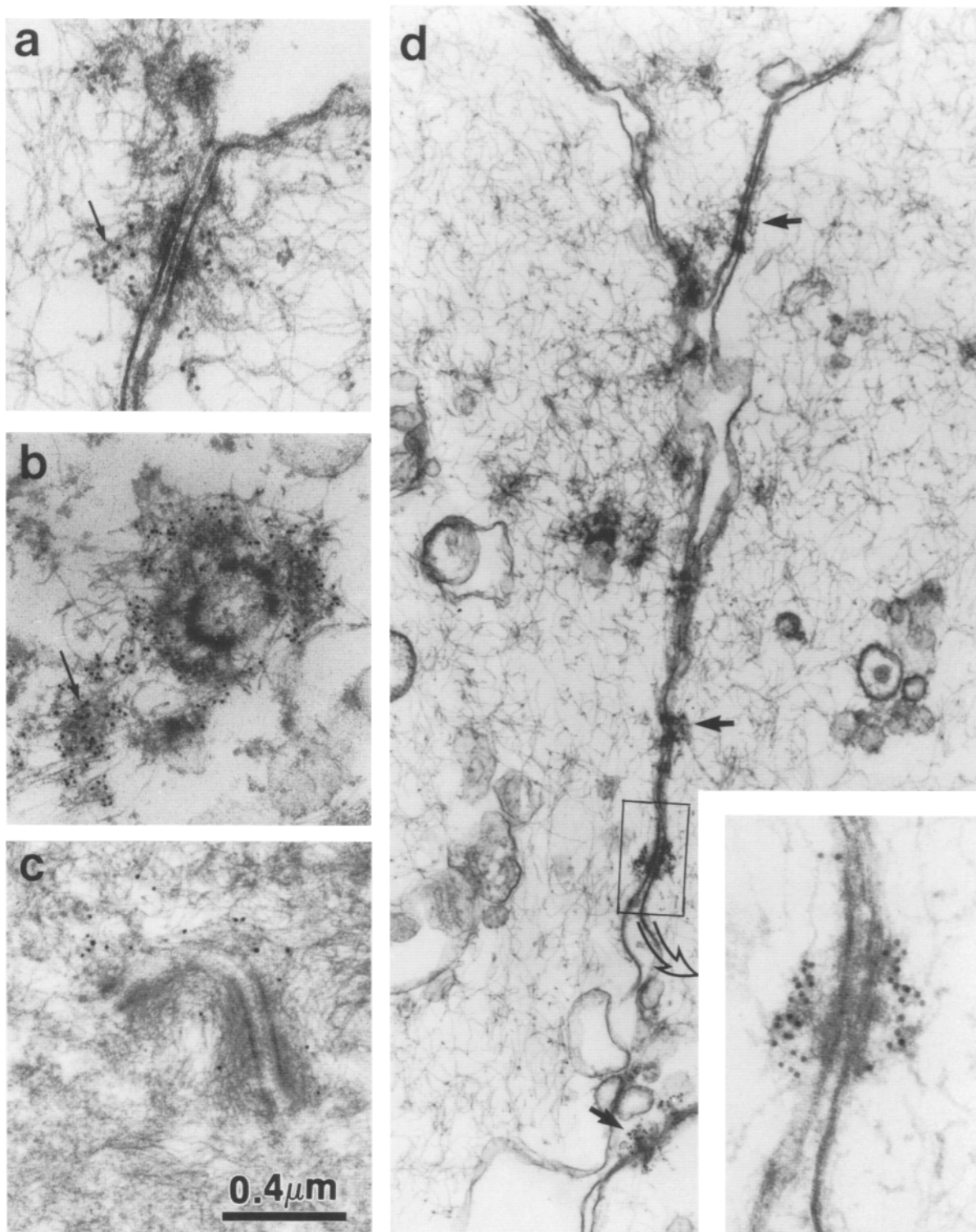


Figure 2. Chick corneal epithelia (17 d, *a*, *b*, and *d*) and MDCK cells (*c*) fixed with 1.5% formaldehyde, were permeabilized with 0.5% Triton-X 100. Subsequent to incubation with 10 nm gold-08L conjugates, the samples were processed for routine EM. Gold particles can readily be seen in the region of desmosomes. Gold particles can be seen to decorate amorphous material on the cytoplasmic face of desmosomes and the intermediate filaments immediately adjacent to the desmosomes (*arrows*), however, the desmosomal plaque proper exhibited little or no staining. In addition, intermediate filaments distant from the desmosome complex bound little or no 08L-gold (*d*). The survey micrograph shown in *d* demonstrates that 08L was localized only to areas in the vicinity of desmosomes, not to other cytosolic structures. Inset in *d* demonstrates higher magnification of a desmosome decorated with 08L-gold.

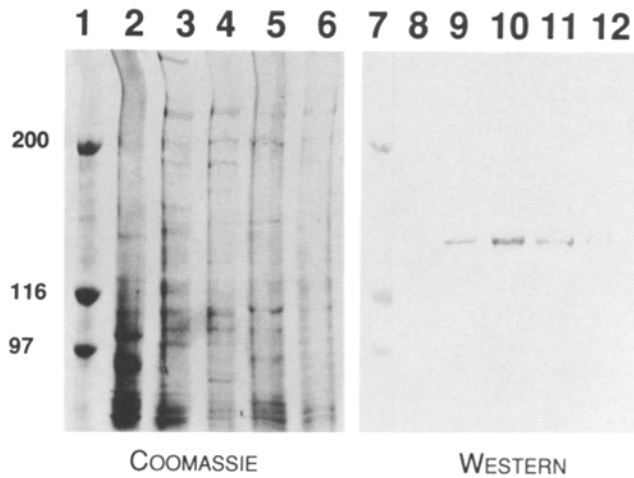


Figure 3. Confluent MDCK cells were extracted with Triton-X 100 (lanes 2, 3, 8, and 9), Triton-X 100 and 1.5 M KCl (lanes 5, 6, 11, and 12), and successively with Triton-X 100 then 1.5 M KCl (lanes 4 and 10). Lanes 2–6 contain Coomassie blue-stained SDS-PAGE and lanes 8–12 demonstrate corresponding immunoblots. Subsequent to the extractions, supernatants and insoluble materials were resolved on SDS-PAGE and immunoblotted. The O8L antigen presented as a 140-kD protein. O8L antigen was found in the Triton-insoluble fractions (lanes 3 and 9), little or no O8L antigen was recognized in Triton-soluble fractions (lanes 2 and 8). KCl extraction of the Triton insoluble material released the O8L antigen (lanes 4 and 10). The O8L antigen was detected in the soluble fractions of MDCK extracted simultaneously with Triton-X 100 and KCl (lanes 5 and 11). The corresponding reduction of insoluble O8L can be seen in lanes 6 and 12.

quently, cultures were switched to NCM and the distributions of O8L antigen and desmoplakin were examined at varied time points. Double immunofluorescence microscopy showed that in LCM, both O8L antigen and desmoplakin were distributed intracellularly. The O8L staining appeared more punctate than that of desmoplakin (Fig. 6, *a* and *a'*), but they did not appear to co-localize. Upon shift to NCM, desmoplakin staining appeared at the lateral cell boundary as early as 15 min, exhibiting strong staining within 2 h (Fig. 6 *b'*). Significant levels of staining for O8L was not observed until after 5 h (Fig. 6 *c*). With longer incubation times, the cytoplasmic staining of O8L gradually diminished with a corresponding increase in peripheral staining. 4-d cultures in NCM showed that O8L staining on MDCK cells was restricted to lateral boundary with little or no cytoplasmic staining (as shown in Fig. 1). These data are suggestive that O8L may assemble to the vicinity of the desmosome after desmoplakin can be detected and after morphologically recognizable desmosomes can be observed (Mattey and Garrod, 1986a). Whether or not these early junctions, which lack O8L, represent mature and stable desmosome structures still remains to be determined.

The calcium switch assays also suggest that O8L may exist either complexed to desmosomes or in punctate locations within the cytoplasm. We next examined the solubility state of O8L during the transition from LCM to NCM conditions. There was no change in the relative abundance of accumulated O8L protein in soluble versus insoluble pools, with the majority of O8L found in the Triton-insoluble material (Fig. 7). While these data do not rule out the possibility that O8L

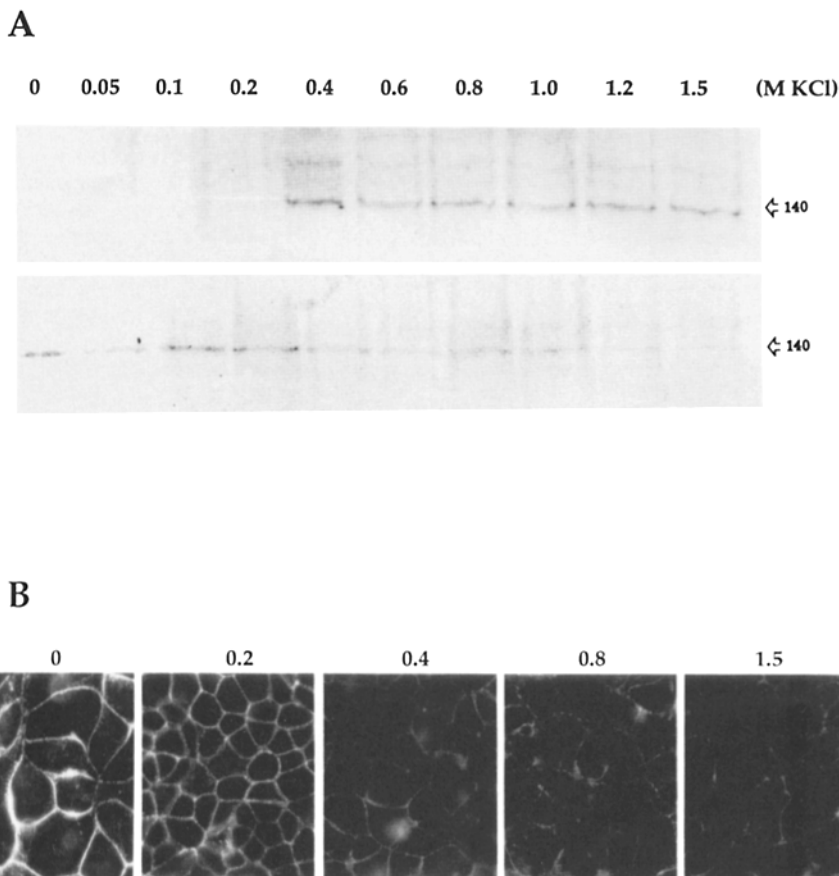


Figure 4. MDCK cells cultured in dishes (*A*) or on coverslips (*B*) were extracted with CSK with different concentrations of KCl for 30 min at 4°C. KCl extracts (*A*, top) or KCl insoluble material (*A*, bottom) were boiled with sample buffer and subjected to SDS-PAGE and immunoblotted. Cells on coverslips after extraction as described above were fixed and permeabilized with acetone and analyzed by immunofluorescence microscopy as described previously (*B*). The O8L antigen was extracted in buffers containing KCl > 0.4 M, and the amount of O8L antigen remaining in the KCl insoluble pellet decreased accordingly. Immunofluorescence microscopy showed that O8L intensity decreased dramatically upon extraction with 0.4 M KCl, compared with those extracted with 0.2 M KCl.

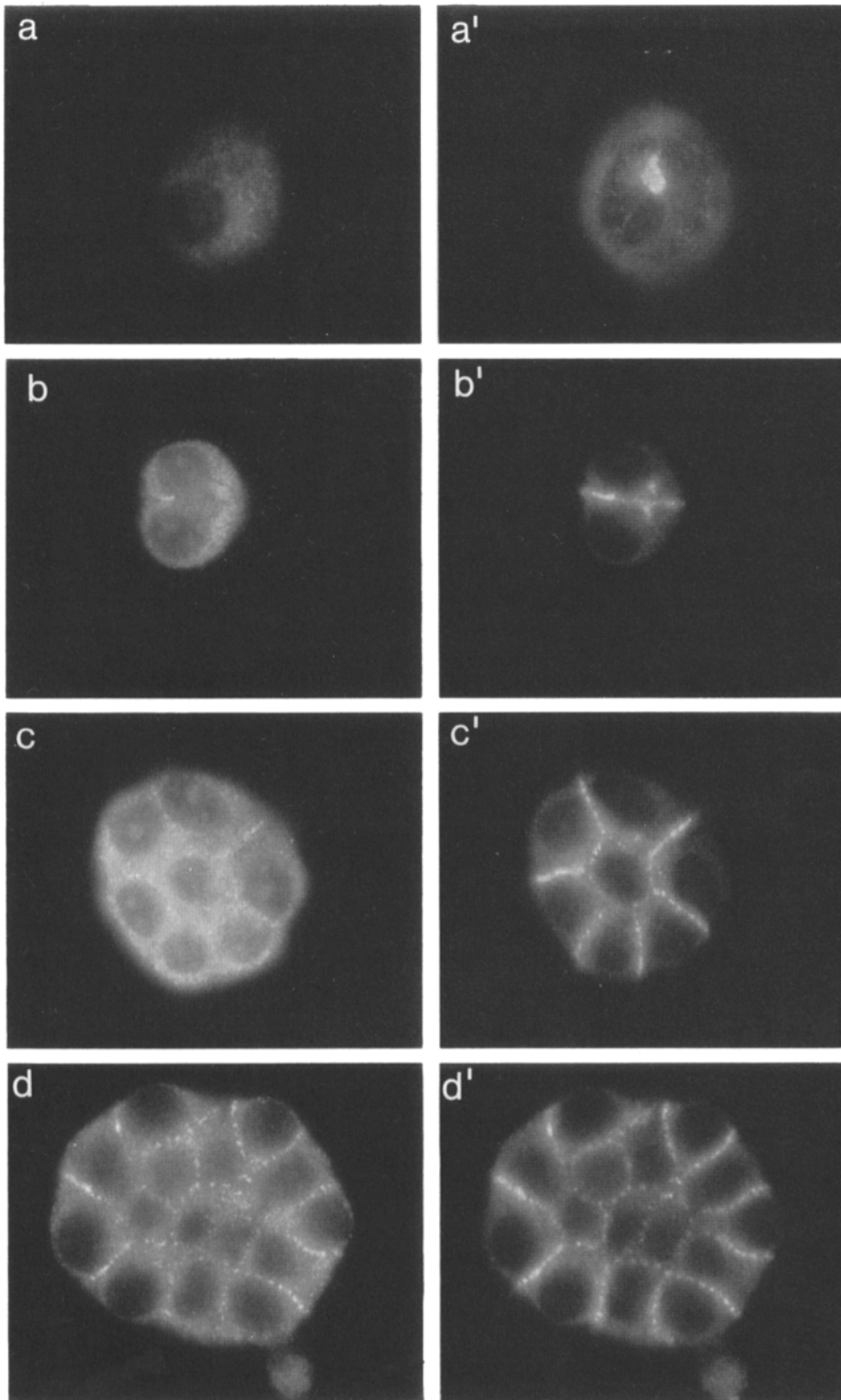
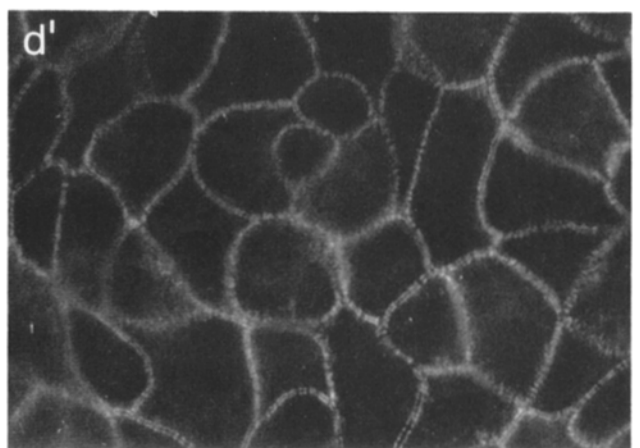
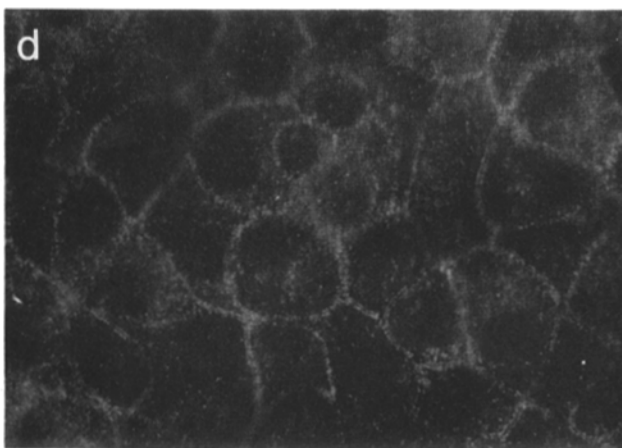
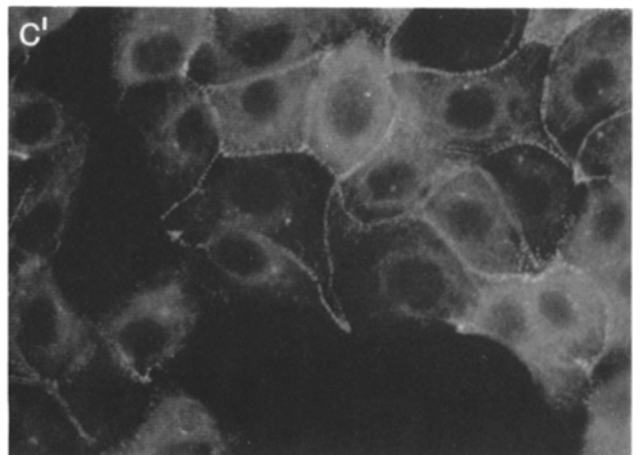
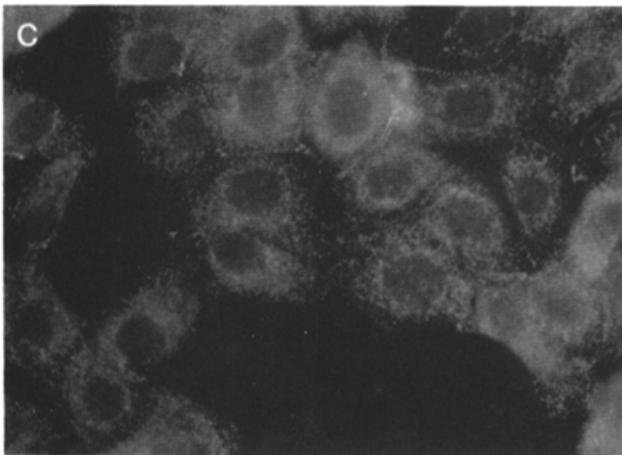
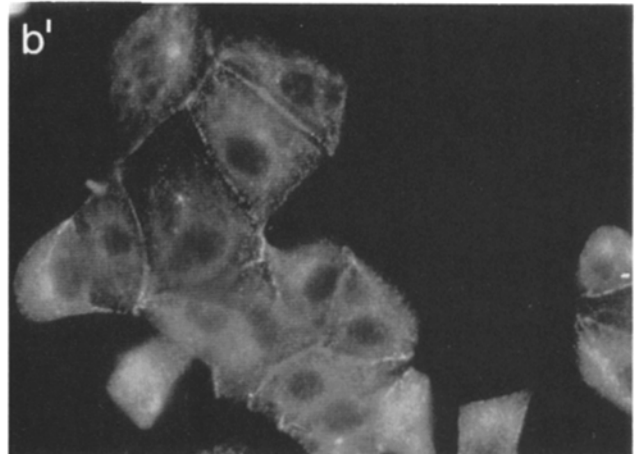
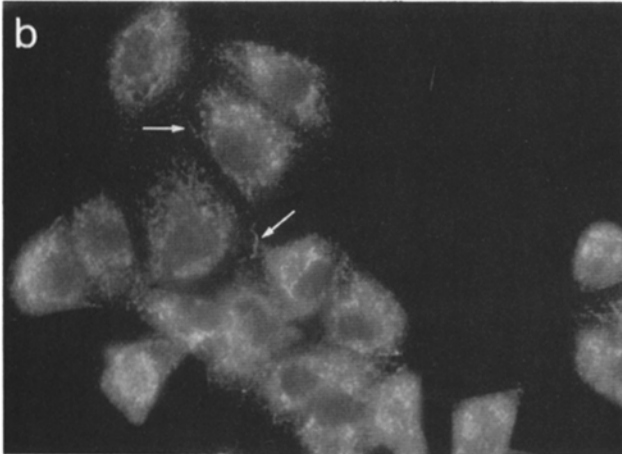
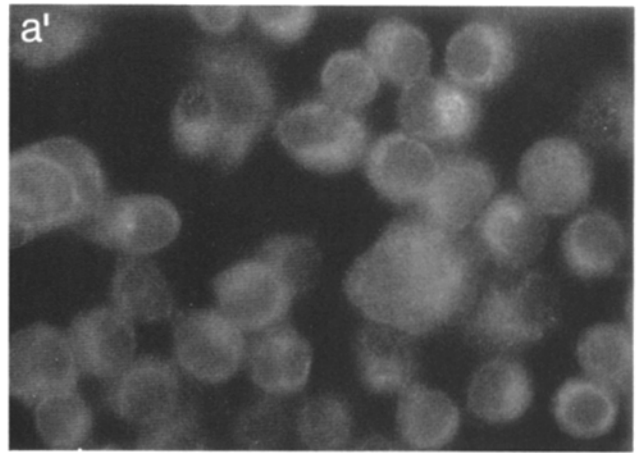
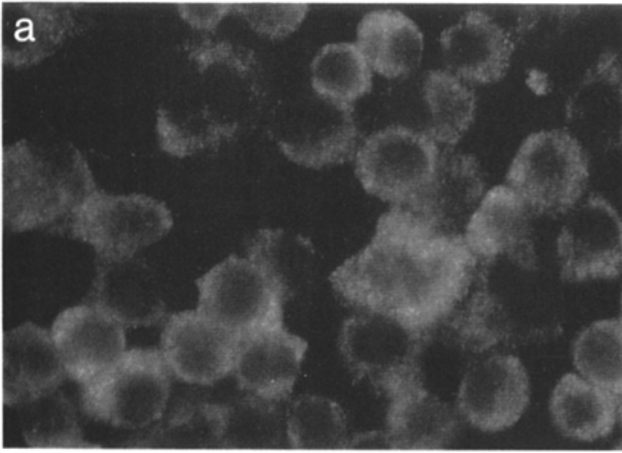


Figure 5. Double immunostaining of low confluence cultures of MDCK cells with 08L antibodies (*a*, *b*, *c*, and *d*) and anti-desmoplakin (*a'*, *b'*, *c'*, and *d'*) demonstrated that, in isolated cells, both 08L (*a*) and desmoplakin (*a'*) were distributed intracellularly, however, they exhibited little or no co-distribution. Desmoplakin was seen within aggregates in a paranuclear location. In two cell aggregates, 08L (*b*) appeared predominantly as intracellular punctate staining with limited staining along cell-cell contact surfaces, whereas, desmoplakin (*b'*) was mostly seen assembled along the lateral contacts. In the larger islands of cells, 08L was seen to co-distribute with desmoplakin at the lateral surfaces with diminished cytosolic staining (*c*, *c'*, *d*, and *d'*).

may exist in the soluble pool with a very short half-life, these data do suggest that 08L may exist intracellularly in an insoluble state. Further in depth biochemical analyses, such as gel filtration and/or sucrose gradient centrifugation of pulse chased material are required to address the solubility state of 08L and associated molecules during desmosome assembly.

Further investigation into the timing of assembly of 08L, desmoplakin, and cytokeratin was carried out. In low calcium conditions the staining pattern of 08L did not seem to co-align with that of cytokeratin (Fig. 8, *a* and *a'*). However, at this resolution it is difficult to absolutely exclude the possibility of 08L cytokeratin co-distribution. Desmoplakin, however, showed some, albeit limited, co-distribution with



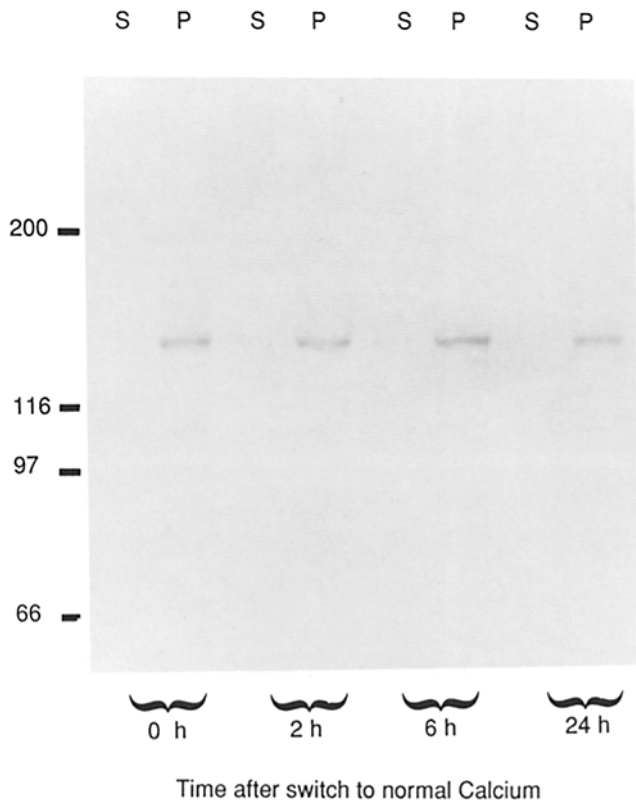


Figure 7. MDCK cells cultured in low calcium medium then transferred to NCM for 0, 2, 6, and 20 h were extracted with Triton-X 100 containing buffers. Subsequent to extraction, Triton-X 100 soluble material (s) and insoluble pellet (p) were analyzed by Western blots with O8L antibody. O8L was found predominantly in the Triton insoluble material.

perinuclear keratin whorls (Fig. 8, *d* and *d'*, arrows). Within 2 h after the switch to normal calcium levels, desmoplakin was seen along cell-cell contact surfaces, whereas O8L staining of lateral surface was limited (Fig. 8, *b* and *e*). Keratin filament immunostaining at 2 h post-switch revealed relatively little organization to the cell boundaries. However, when cytokeratin filaments were seen to be organized perpendicularly to lateral surfaces, immunostaining for O8L could be detected at the cell margin (Fig. 8, *b* and *b'*). The cell margin immunostaining pattern for desmoplakin, however, did not seem to correlate with perpendicularly arranged cytokeratin filaments (Fig. 8, *e* and *e'*). While most sites of cell-cell contact decorate with desmoplakin staining, the majority of the desmoplakin staining did not appear to correlate with anchorage for perpendicularly arranged intermediate filaments. Cells immunostained 5 h after switching

revealed more O8L assembled to lateral surface and extensive perpendicularly organized keratin filament bundles (Fig. 8, *c* and *c'*). While these data tempt one to suggest a correlation between the presence of O8L to the lateral cell boundary and the higher organization of the keratin cytoarchitecture, more in depth studies including immunogold EM will be necessary to confirm the role of O8L in the organization of the epithelial cytokeratin scaffold.

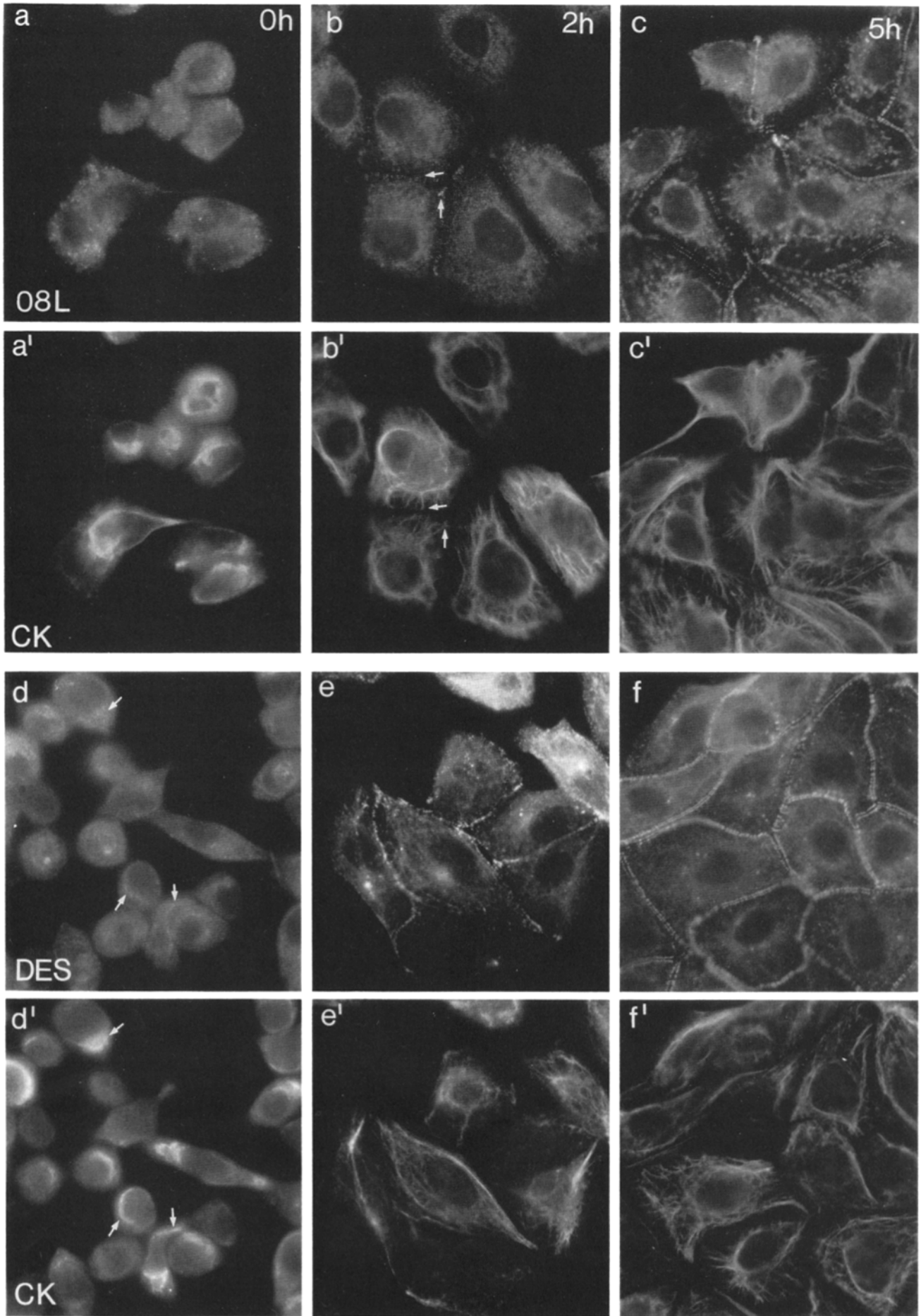
Discussion

Cell-cell adhesion is widely believed to be a fundamental process in the development of multicellular organisms. Junctions of the adherens type are of particular interest and integral to our understanding of cell and tissue biology, because they play a role in not only adhesion of epithelial cells but also in the organization of the epithelial cytoskeleton (Cowin et al., 1985a; Geiger and Ginsberg, 1991; Bologna et al., 1986). In this work, we present data regarding the identification of a novel protein $M_r \approx 140,000$, which is found within most, if not all, epithelial cells and is related to the macula adherens junction (desmosome). Double staining with desmoplakin antibodies localized O8L to, or near, the desmosome. Electron microscopic analyses of MDCK cells and corneal epithelia confirmed the O8L protein location to the cytoplasmic face of desmosomes.

The known molecular components of the desmosome proper have been examined in much detail (for reviews see Cowin et al. 1985a; Schwartz et al., 1990; Garrod et al., 1990; Jones and Green, 1991). The O8L protein does not seem to be identical to any known desmosomal proteins. However, there have indeed been reports of desmosome associated proteins with relative molecular masses around 140,000. These include $M_r \approx 125-140,000$ cell surface glycoproteins which may be cell adhesion molecules (Jones et al., 1986; Jones, 1988). In addition, Cartaud and co-workers (1990) have described a 140-kD desmosomal protein which binds intermediate filaments and is antigenically related to lamin B. Although the O8L protein described here may be similar or related to the protein reported by Cartaud et al. (1990), the O8L protein, unlike the lamin B-related protein, does not localize to the desmosomal plaque proper. Also, the 140-kD lamin B-related protein has a distinctly acidic PI of 5.4 with no indication of isoforms. The O8L protein displays isoforms from PI 5.9-6.2 (data not shown). While our studies suggest that O8L protein and the 140-kD protein described by Cartaud and colleagues (1990) may be distinct, definitive resolution of this point awaits more direct comparisons and/or primary sequence analysis.

The location of the O8L protein at the interface between the desmosome proper and IFs may be suggestive that O8L is involved in the interaction between IFs and the desmo-

Figure 6. MDCK cells cultured in LCM, after 2 d, were shifted to NCM. Cells were double immuno-stained with O8L (*a*, *b*, *c*, and *d*) and anti-desmoplakin (*a'*, *b'*, *c'*, and *d'*) after 0, 2, 5, 20 h of incubation in NCM. MDCK cells in LCM exhibited no obvious cell-cell contacts and O8L and desmoplakin were distributed intracellularly (*a* and *a'*, respectively), with little or no co-distribution. After shifting to NCM, staining for desmoplakin can be seen at the lateral cell surface within 2 h (*b'*). The O8L antigen, showed limited cell margin staining (*b*, arrows) until around 5 h (*c*). After 20 h in NCM both O8L and desmoplakin can be located along the lateral cell margins with decreased cytosolic staining (*d* and *d'*).



some. Thus far, a number of desmosomal components have been described as putative linker molecules connecting IF to the desmosome. For example, desmoplakin I and II are components of the desmosomal plaque and have been shown to associate with IF during desmosome formation and assembly (Mueller and Franke, 1983; Jones and Goldman, 1985). In addition, sequence information of desmoplakin predicts that the carboxyl terminus consists of a globular domain with basic and acidic residue periodicity complementary to the rod domain of IFs (Green et al., 1990). However, studies using a variety of in vitro assays have been unable to demonstrate direct binding of desmoplakin to IF (O'Keefe et al., 1989). It has been suggested that desmoplakin-IF binding may require the involvement of additional accessory proteins (Jones and Green, 1991; Stappenbeck and Green, 1992).

To gain insight into the 08L-antigen association with the desmosome, we chose to examine desmosome-IF complex assembly. In low calcium conditions both desmoplakin and 08L antigen were localized to the cytosol with little or no co-alignment. After changing cells from low calcium to normal calcium medium, desmoplakins were seen to assemble to cell-cell boundaries significantly before the 08L protein. The timing of desmosome reformation after Ca^{2+} switch in our system, was consistent with that reported in previous studies (Mattey and Garrod, 1986a). We have also found that as MDCK cells advanced from low density to higher density, the temporal sequence of assembly of 08L protein and desmoplakin to the cell-cell contact boundaries was the same as seen in the Ca^{2+} switch assays. These data are suggestive that the assembly of 08L protein to the lateral cell boundary occurs subsequent to desmosome formation. Therefore, we suggest that the 08L antigen most likely does not function as the primary IF-desmosome linker but may be involved in the assembly and/or organization of the intermediate filament network.

The assembly of desmosomes by cultured epithelial cells has been extensively studied (Overton, 1962; Lentz and Trinkhaus, 1971; Jones and Goldman, 1985; Mattey and Garrod, 1986a; Duden and Franke, 1988; Pasdar and Nelson, 1988a,b, 1989; Pasdar et al., 1991). It is clear that certain epithelial systems, such as the MDCK cell line, cells in low calcium conditions (LCM) possess all the requisite components for desmosome formation (Mattey and Garrod, 1986a; Pasdar and Nelson, 1988a, 1989). So that, when the calcium level is restored there is a rapid assembly of well formed desmosomes. We have shown that in low calcium conditions, 08L and desmoplakin may be sequestered in distinct locations within the cytosol similar to the previous observations on desmoglein and desmoplakin (Pasdar et al., 1991).

Mattey and Garrod (1986b) have described the formation of stable desmosomal linkages in MDCK cells. They showed that although morphologically "mature" desmosomes may be established within the first hour in normal calcium concentrations, these desmosomes remain sensitive to low Ca^{2+} conditions for some time. They suggest that additional accessory desmosomal components reinforce the desmosome and result in a stable, low Ca^{2+} insensitive, epithelial adhesion. The recruitment of 08L protein to the lateral membrane was correlated with a more ordered array of the intermediate filament associating with desmosomes. It is tempting to speculate that the 08L protein may somehow contribute to the stability of the desmosomal junction.

Cycloheximide treatment does not interfere with 08L assembly to the desmosome-IF complex, indicating de novo protein synthesis is not required and that functional 08L antigen may exist in a cytosolic pool in low calcium condition (data not shown). The gross solubility of 08L protein does not appear to change in cells under low or normal calcium conditions, suggesting that 08L antigen is aggregated or complexed with other cytosolic proteins. However, we have shown that 08L does not co-localize with desmoplakin nor cytokeratin under these conditions. Therefore, although 08L is eventually recruited to the desmosome, it is not located in preformed association with desmoplakin.

In this paper, we identify a novel protein localized to the region of the IF-desmosome complex on the lateral epithelial surface. This protein is not identical to any desmosomal proteins nor to known IFs associated proteins. Based on its location between desmosomal plaque and associated bundles of IFs, we postulate that this molecule may be involved in the association of IFs to the desmosome structure. Identification of such molecules will contribute to our current understanding of the functions of the IF-desmosome complex in epithelial adhesion, and the role of specific cell surface domains such as desmosomes in the integration and organization of epithelial cytoskeleton.

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Figure 8. Cells cultured in low calcium medium (*a*, *a'*, *d*, and *d'*), 2 h after shifting to NCM (*b*, *b'*, *e* and *e'*) or 5 h after shifting to NCM (*c*, *c'*, *f*, and *f'*) were double immunostained with 08L (*a-c*), anti-cytokeratin (*a'-c'* and *d'-f'*) or with desmoplakin antibodies (*d-f*). In LCM, cells exhibited punctate cytosolic 08L staining, whereas cytokeratin was concentrated in whorls around the nucleus. Cells cultured for 2 h in NCM demonstrated limited assembly of 08L to the lateral cell surface (*b*) with some organization of the cytokeratin network (*b'*) notably in areas where 08L was located at the cell margin, where the keratin filaments can be seen arranged perpendicular to the cell surface (*arrows*). 08L staining at the lateral surfaces was marked at 5 h after shifting to normal calcium levels. Here, extensive organization of the keratin filaments can be seen with convergence of filaments toward areas of the cell surface decorated with 08L (*c* and *c'*). Desmoplakin and cytokeratin were seen partially co-distributed in cells cultured in low calcium medium (*d* and *d'*). At 2 h after shifting to normal calcium medium, desmoplakin was seen at most cell-cell contact surfaces but there was little organization of the keratin network (*e* and *e'*). After 5 h in NCM, desmoplakin was found along cell borders with diminished cytosolic staining. Extensive reorganization of cytokeratin filament can be seen (*f* and *f'*).

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