Kalinin: An Epithelium-Specific Basement Membrane Adhesion Molecule That Is a Component of Anchoring Filaments

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Abstract. Basal keratinocytes attach to the underlying dermal stroma through an ultrastructurally unique and complex basement membrane zone. Electron-dense plaques along the basal surface plasma membrane, termed hemidesmosomes, appear to attach directly to the lamina densa of the basement membrane through fine strands, called anchoring filaments. The lamina densa is secured to the stroma through a complex of type VII collagen containing anchoring fibrils and anchoring plaques. We have identified what we believe is a novel antigen unique to this tissue region. The mAbs to this antigen localize to the anchoring filaments, just below the basal-dense plate of the hemidesmosomes. In cell culture, the antigen is deposited upon the culture substate by growing and migrating human keratinocytes. Addition of mAb to the cultures causes the cells to round and detach, but does not impair them metabolically. Skin fragments incubated with antibody extensively de-epithelialize. These findings strongly suggest that this antigen is intimately involved in attachment of keratinocytes to the basement membrane.

This antigen was isolated from keratinocyte cultures by immunoaffinity chromatography. Two molecules are observed. The most intact species contains three nonidentical chains, 165, 155, and 140 kD linked by interchain disulfide bonds. The second and more abundant species contains the 165- and 140-kD chains, but the 155-kD chain has been proteolytically cleaved to 105 kD. Likewise, two rotary-shadowed images are observed. The larger of the two, presumably corresponding to the most intact form, appears as an asymmetric 107-nm-long rod, with a single globule at one end and two smaller globules at the other. The more abundant species, presumably the proteolytically cleaved form, lacks the distal small globule. We propose the name "kalinin" for this new molecule.

THE basement membrane zones at epithelial–stromal interfaces of external tissues are unique and complex. The dermal–epidermal junction is one of the best studied (Palade and Farquhar, 1965; Rowlatt, 1969; Susi, 1967; Bruns, 1969; Briggaman and Wheeler, 1975*a*; Kawanami et al., 1978). When visualized by EM following standard conditions of fixation, dehydration, and staining, a typical basal lamina is seen, containing a lamina lucida and lamina densa extending ~100 nm from the epithelial basal surface (see Fig. 2 *a*). In addition, electron-dense thickenings, termed hemidesmosomes (Weiss and Ferris, 1954; Kelly, 1966), are seen along the basal keratinocyte surface. Thin filaments, termed anchoring filaments (Ellison and Garrod, 1984), appear to transverse the lamina lucida, bridging the hemidesmosome and the lamina densa. Along the dermal margin of the lamina densa, anchoring fibrils extend from the basement membrane. The anchoring fibrils either loop back and reinsert into the lamina densa, or extend perpendicularly from the basement membrane and insert into anchoring plaques, which are electron-dense condensations of the ends of anchoring fibrils together with other intrinsic constituents of the basal lamina (Keene et al., 1987; Gipson et al., 1987). The ultrastructure of the anchoring fibril network suggests that it secures the basement membrane to the underlying dermis (Susi et al., 1967; Kawanami et al., 1978). This hypothesis is supported by observations that individuals with recessive dystrophic epidermolysis bullosa lack anchoring fibrils (Briggaman and Wheeler, 1975*b*; Leigh et al., 1988; Bruckner-Tuderman et al., 1989), and suffer from spontaneous separation of the epidermal basement membranes from the subadjacent stroma.

The ultrastructural heterogeneity of the dermal–epidermal junction is reflected by the presence of several glycoproteins whose localization is limited to this zone. Hemidesmosomes contain several proteins (Mutasim et al., 1989; Westgate et al., 1985; Reignier et al., 1985; Jones et al., 1986; Stanley et al., 1984; Labib et al., 1986; Mueller et al., 1989) that demonstrate M_r values from 165,000–240,000. The integrin $\alpha 6\beta 4$ has also been recently localized to the external region of the hemidesmosome (Stepp et al., 1990). The anchoring fibrils have been determined to consist of lateral unstaggered aggregates of type VII collagen (Sakai et al., 1986*a*; Lunstrum et al., 1987). In addition to these proteins, several antibodies recognizing antigens unique to the dermal–epidermal

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junction have been used for clinical diagnosis of epidermolysis bullosa, even though the antigens are only partially characterized and the ultrastructural correlates are not known (Fine, 1988).

The structural relationships between proteins of the hemidesmosomes, the ubiquitous intrinsic basement membrane constituents, and type VII collagen in anchoring fibrils is not clear. Ultrastructural analyses of this zone suggests direct connections between the anchoring fibrils and the hemidesmosomes (Susi et al., 1967; Ellison and Garrod, 1984). The association of anchoring fibrils and hemidesmosomes is also suggested by the observations that in vitro, hemidesmosomes regenerate only on the sites along denuded basement membranes directly over anchoring fibrils (Gipson, 1983) and that hemidesmosomes and anchoring fibrils appear simultaneously during development and wound healing (Gipson et al., 1988; Smith et al., 1988). However, our published model for the structure of anchoring fibrils predicts that direct contact between type VII collagen and the hemidesmosome is unlikely (Lunstrum et al., 1987; Keene et al., 1987; Bächinger et al., 1990). The NC-1 domain of type VII collagen has a length of only 37 nm. If the standard EM image truly reflects the in vivo structure of the dermal–epidermal junction, then the NC-1 domain is not large enough to span the entire basement membrane. This model predicts that additional proteins are likely to link type VII collagen to the hemidesmosomal proteins.

To address the above hypothesis, the present study is the description of a protein identified by a search for the anchoring filament protein. The ultrastructural location, the filamentous conformation, and the tissue distribution support this identification. The protein is shown to be necessary for the in vitro attachment of keratinocytes to plastic or glass substrates and to the basement membrane in vivo. We propose the name "kalinin" for this protein.

Materials and Methods

Antibodies

The BM165 immunogen was derived from an extract of human amnion, prepared as follows. Collagenase extraction and purification of the NC-1 globular domain of type VII collagen from human amnion has been described previously (Bächinger et al., 1990). During one step of this purification, the extract is incubated with DEAE-cellulose (DE52; Whatman Laboratory Products Inc., Clifton, NJ) in a low salt buffer (2 M urea, 25 mM NaCl, 5 mM EDTA, and 50 mM Tris-HCl, pH 7.8). This unbound fraction was used in the further purification of the NC-1 domain. The DEAE was washed with an equal volume of buffer containing 0.2 M NaCl and the eluted material was isolated after centrifugation (17,000 *g* for 60 min). The sample was concentrated 10-fold by ammonium sulfate precipitation (50% saturation) and equilibrated in PBS by dialysis. The resulting complex mixture of proteins served as immunogen.

Hybridomas were prepared and screened by indirect immunofluorescence as previously described (Sakai et al., 1986*a*). The BM165 mAb, an IgG_1, was purified from cell culture supernatants as described elsewhere (Keene et al., 1990). The mAbs 1ID5 (Engvall et al., 1990) and 4C7 (Engvall et al., 1986*a*) specific for the laminin A chain, 4E10 (Wewer et al., 1983) specific for the B chain, and 5C6 (Engvall et al., 1986*b*) specific for type VI collagen were kindly provided by Dr. Eva Engvall of the La Jolla Cancer Research Foundation (La Jolla, CA). Rabbit polyclonal antiserum against mouse laminin was obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

Human foreskin keratinocytes were prepared according to published procedures (Boyce and Ham, 1985). Cells were grown in Keratinocyte Growth Medium containing 0.15 mM $CaCl_2$ and subcultured according to the manufacturers instructions (Clonetics, San Diego, CA). For most immunocytochemical experiments first or second passage cells were grown in glass or plastic chamber slides (Lab-Tek Div. Miles Laboratories Inc., Naperville, IL) or on glass coverslips to ~80% confluency. For large scale collection of spent media cells were grown in 150-cm^2 tissue culture dishes and fed three times per week with 15-ml fresh media.

Human foreskin fibroblasts were generated from explant culture and cultivated by standard techniques in DME containing 10% FBS.

Cell Attachment and Detachment Assays

Cell attachment experiments were performed in 24-multi-well plates (Costar Data Packaging, Cambridge, MA) using untreated plastic or an endogenous keratinocyte matrix. The matrix was generated following solubilization of confluent cell layers with a solution containing 1% Triton-X-100, 10 mM EDTA, and 25 mM Tris-HCl, pH 7.5. The matrix was washed extensively with PBS before use. For attachment, suspensions of keratinocytes (1×10^5 cells/well) in growth medium and fibroblasts (2.5×10^5 cells/well) in serum-free DME were incubated for 1 h at 37°C with the indicated additions. For detachment, cells were plated in untreated wells for 24 h in complete medium, washed, and incubated with the indicated additions for 4 h at 37°C. Quantitation of attached cells was performed essentially as described previously (Aumailley et al., 1989), except that the stain was solubilized with 4 M guanidine before measuring absorbance at 570 nm.

Affinity Purification of the BM165 Antigen

Media collected from growing keratinocytes was clarified by centrifugation (2,000 *g* for 10 min) and endogenous protease activity was minimized by the addition of EDTA, PMSF, and *N*-ethylmaleimide to final concentrations of 5 mM, 50 μM, and 50 μM, respectively. The media was sterilized by filtration and either processed immediately or stored frozen at −20°C until use. The BM165 mAb was conjugated to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ), at 1 mg of antibody per ml of resin, as described by the manufacturer. Keratinocyte media (1–2 liters) was passed through a 15-ml antibody column and the column was washed with PBS. The antigen was eluted with 1 M acetic acid and fractions were monitored for absorbance at 280 nm. Pooled fractions were treated with diisopropylfluorophosphate (5 μg/ml) and dialyzed into appropriate buffers for further analysis. For SDS-PAGE samples were separated on 3–5% gradient gels before reduction and on 5% gels after reduction with β-mercaptoethanol. In addition to high molecular weight prestained standards (Biorad Laboratories, Richmond, CA), the disulfide bonded type VII collagen NC-1 domain (450,000 M_r), reduced NC-1 (M_r 150,000), and reduced fibrillin (350,000 M_r; Sakai et al., 1986*b*) were used in determining relative molecular weight scales.

EM

Tissue. Enbloc immunolocalization of antigens was performed as previously described (Keene et al., 1987) with some modification. Briefly, human neonate foreskin collected shortly after circumcision was cut into 0.5-mm × 1-mm blocks, all including epithelium, and washed for 2 h in PBS, pH 7.4, at 4°C. They were then incubated in primary antibody (200 μg/ml) in PBS overnight at 4°C, rinsed in several changes of PBS over 6 h, and then incubated in 1-nm gold–conjugated secondary antibody (Janssen Life Sciences Products, Piscataway, NJ) diluted 1:3 in PBS containing 1.0% BSA overnight at 4°C. After washing, tissues were submersed in ice-cold silver intensification solution (Janssen Life Sciences Products) for 15 min, and then rapidly warmed to room temperature. After allowing silver to precipitate upon the 1-nm gold particles for 7 min at room temperature, the tissues were rinsed several times over 15 min with water, and then with 0.1 M cacodylate buffer, pH 7.4. Tissues were finally fixed in 0.1 M cacodylate buffered 1.5%:1.5% glutaraldehyde/paraformaldehyde, pH 7.4, dehydrated in a graded series of ethanol dilutions, exposed to propylene oxide, and embedded in Spurrs epoxy. Control antibodies used included those recognizing elastin (mAb 10B8, produced and generously provided by Dr. Lynn Sakai, Shriner's Hospital), collagen type IV (Sakai et al., 1982), and collagen type VI (Engvall et al., 1986*b*). One sample of skin was fixed for 30 min in ice-cold acetone, rinsed in buffer, further fixed in 3%:3% aldehydes and 1% OsO4, and then dehydrated in acetone before embedding in Spurrs epoxy to demonstrate the presence of anchoring filaments (see Fig. 2 *a*).

Cells. For examination of normal cell ultrastructure before antibody treatment, human keratinocyte cultures were grown on glass coverslips and fixed in 0.1 M cacodylate buffered 1.5%:1.5% glutaraldehyde/paraformal-

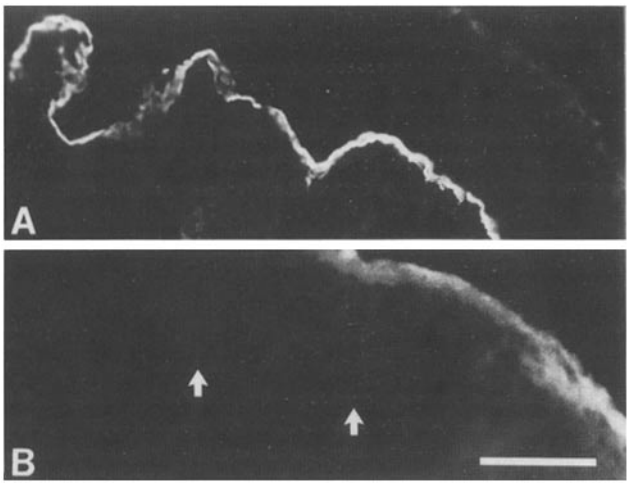

Figure 1. Indirect immunofluorescent localization of the BM165 antigen in human skin. Frozen sections of newborn foreskin were stained with either BM165 (*a*) or media from unfused myelomas (*b*). The BM165 mAb reacts specifically with the dermal–epidermal basement membrane and does not recognize various other basement membranes present in the dermal layer. Arrows indicate the position of the unstained basement membrane. Bar, 100 μm.

dehyde, 1.0% OsO4, dehydrated in a graded ethanol series, and then either embedded directly in Spurrs epoxy for transmission EM (TEM),[1] or critical point dried and sputter coated for scanning EM (SEM) as previously described (Keene et al., 1988). TEM immunoelectron microscopy was performed on keratinocytes grown on eight-well permanox culture flasks using an identical protocol as that described above for tissues, except that the incubation time in primary antibody was for 4 h at room temperature, the secondary antibody was conjugated to 5-nm gold and diluted 1:3 in BSA buffer (20 mM Tris-HCl, 0.9% NaCl, 1 mg/ml BSA, 20 mM NaN3), and the silver intensification procedure was omitted. Keratinocytes grown on glass coverslips and observed by SEM after exposure to antibody were treated identically, except they were critical point dried from liquid CO_2 after dehydration in ethanol.

For routine TEM examination, 60–90-nm thick sections were cut on an ultramicrotome (Reichert Jung, Vienna, Austria) using diamond knives and examined using a microscope (410 L8; Philips Electronic Instruments, Inc., Mahway, NJ) operated at 60 KV after contrasting in uranyl acetate and Reynolds lead citrate (Reynolds, 1963). For routine SEM examination, samples were sputter coated with a minimum amount of Gold-Palladium and observed in the upper stage of a SEM (model DS130; International Scientific Instruments, Inc., Milpitas, CA) operated at 10 kV, using a spot size of 3–10 nm.

Other Techniques

Methods including Western blotting, rotary shadow analysis, and length measurements have been detailed elsewhere (Morris et al., 1986; Lunstrum et al., 1986; Bächinger et al., 1990).

Results

mAbs were raised to a mixture of partially purified proteins originally extracted from human amnion by collagenase digestion as described for the isolation of the type VII collagen NC-1 domain (Bächinger et al., 1990). Resulting hybridomas were screened by indirect immunofluorescence for localization to the dermal–epidermal, but not to the vascular basement membrane zone of human fetal foreskin. Selected hybridomas were rescreened by Western blotting of the immunogen and protein extracts containing known basement membrane components. Hybridomas that did not recognize known basement membrane components were retained for further study. One of these screenings produced two hybridomas that appeared to recognize the same unique protein. One of these, termed BM165 was used for the studies reported here. BM165 specifically identifies the dermal–epidermal junction basement membrane zone of skin, but shows no reactivity to the basement membranes of the vasculature or surrounding nerves (Fig. 1). On salt-split skin the BM165 antigen remains associated with the dermal layer, distinguishing it from both the bullous pemphigoid antigen and the $\alpha 6\beta 4$ integrin which partition with the epidermal layer (not shown). Additionally, the BM165 mAb does not immunoprecipitate laminin or type VII collagen (not shown). The tissue distribution of BM165 reactivity is shown in Table I. All of the subepithelial regions showed crisp, brilliant, continuously linear fluorescence. The tissue distribution directly parallels the occurrence of hemidesmosomes and anchoring fibrils, with the exception of the occasional and weak staining of the intestinal smooth muscle.

The BM165 antibody was then used to localize the antigen within the dermal–epidermal basement membrane of human foreskin. Primary antibody was localized using secondary antibody conjugated to 1-nm gold, which was visualized by silver enhancement. The use of 1-nm gold proved necessary because of the limited penetration of the basement membrane by a 5-nm gold–conjugated secondary antibody. This procedure localizes the BM165 antigen to the anchoring filaments, just below the basal-dense plate of hemidesmosomes (Fig. 2, *b* and *c*). No labeling of the anchoring filaments was seen when an antibody of irrelevant specificity (see Materials and Methods) was used as the primary antibody (data not shown). Some additional label is seen along the lamina densa (Fig. 2 *c*), but the majority of the label underlies the hemidesmosomes. Small amounts of gold deposits are also seen beneath the dermal side of the lamina densa.

Throughout these experiments, extensive, sometimes complete de-epithelization of skin samples during incubation with the primary antibody was commonly observed. This was entirely outside our considerable experience with antibodies to types IV and VII collagens. The regions of unsplit basement membrane shown in Fig. 2, *b* and *c* are those relatively removed from the tissue edge. Near the tissue edge, where the antibody concentration was highest and the epidermis had separated from the basement membrane, very

Table I. The Tissue Distribution of the Antigen Recognized by mAb BM165 as Determined by Indirect Immunofluorescence

Tissue	Result
Skin, subepithelial	+
Trachea, subepithelial	+
Esophagus, subepithelial	+
Cornea, subepithelial	+
Amnion, subepithelial	+
Intestinal smooth muscle	±
Kidney	–
Blood vessels	–
Nerve	–
Cartilage	–

1. *Abbreviations used in this paper*: SEM, scanning EM; TEM, transmission EM.

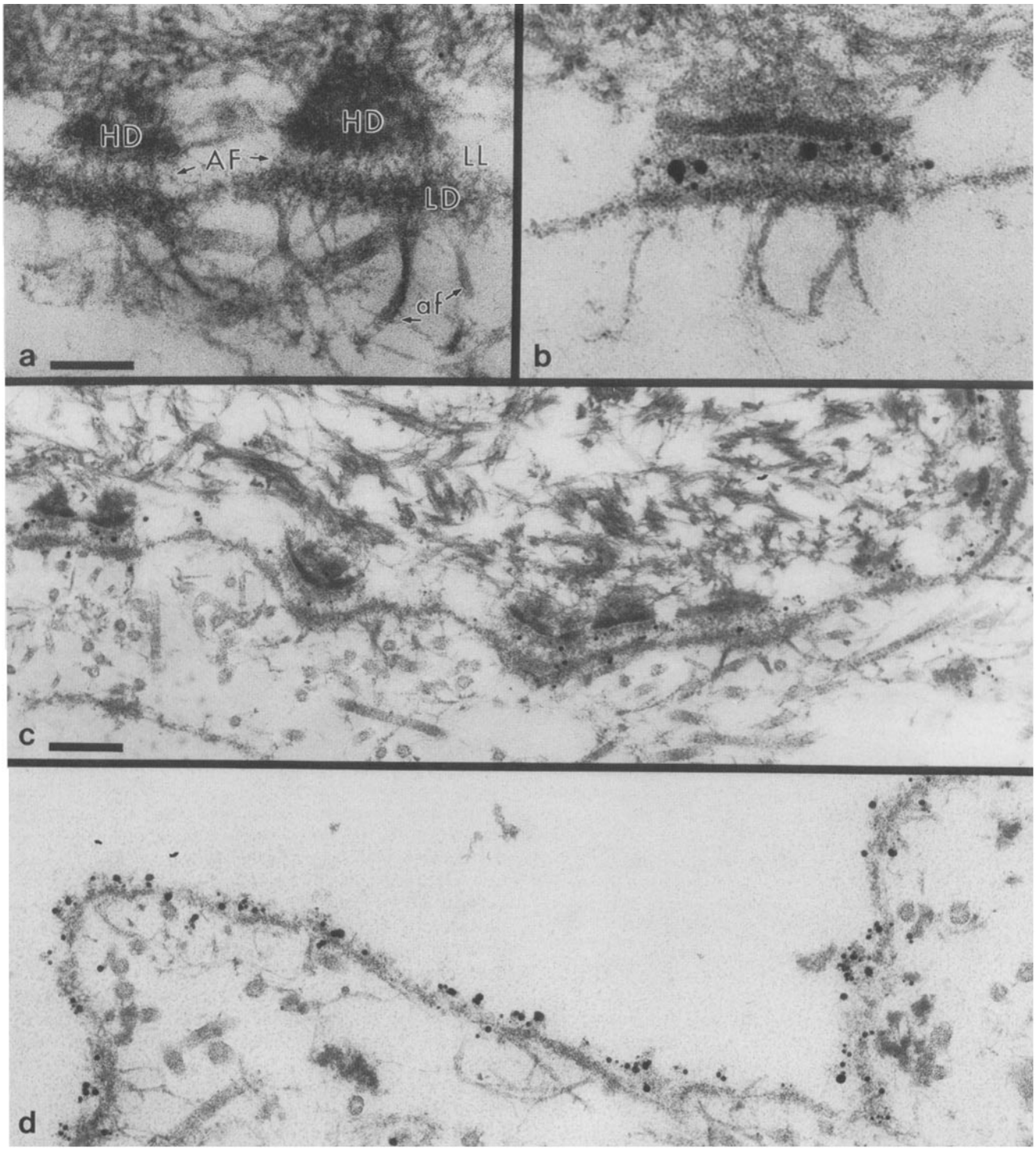

Figure 2. Ultrastructural immunolocalization of the BM165 antigen in human skin. (*a*) Human skin fixed in acetone before aldehyde, allowing the identification of the lamina lucida (*LL*), lamina densa (*LD*), hemidesmosome (*HD*), anchoring filaments (*AF*), and anchoring fibrils (*af*). (*b*) Localization of BM165 mAb using silver-enhanced 1-nm gold–conjugated secondary antibody. The label appears directly over anchoring filaments. (*c*) Lower magnification showing BM165 labeling along a continuous stretch of intact skin and (*d*) along the basement membrane in a region where the antibody has induced epidermal detachment. Bars: *a* and *b*, 100 nm; *c* and *d*, 200 nm.

strong labeling was seen along the lamina densa, at what had been the cell interface (Fig. 2 *d*). Some label was seen still attached to the extracellular face of the hemidesmosome, but this was relatively rare (not shown).

The BM165 antibody was used to visualize the antigen in keratinocyte cultures. As shown in Fig. 3 *a*, when applied to confluent cells, the antibody localizes to the surface of the plastic substrate between the cultured cells (compare to Fig. 3 *c*). No intracellular fluorescence is observed. This unusual localization could not be duplicated with antibodies to type

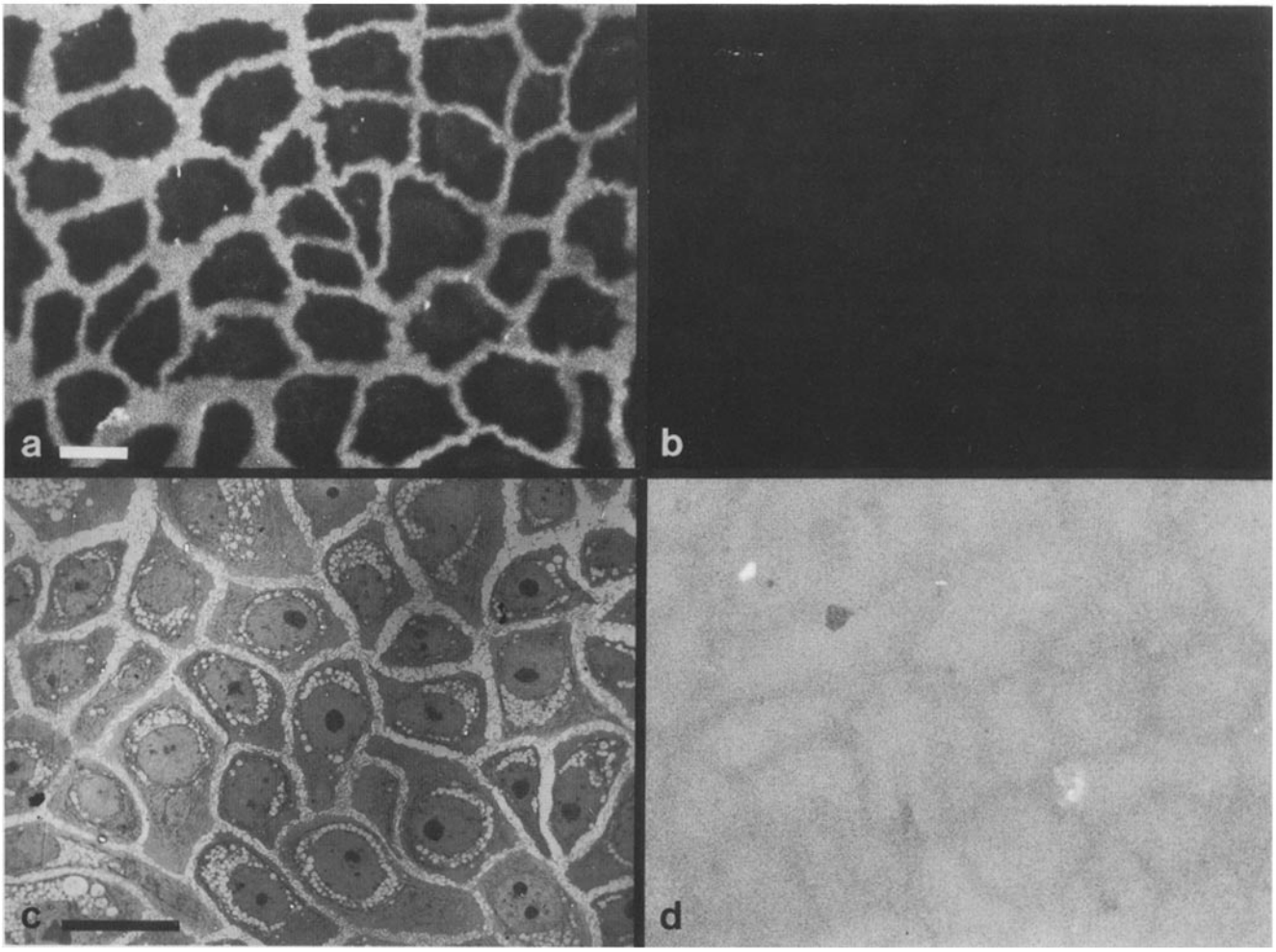

Figure 3. Keratinocytes deposit the BM165 antigen on the culture substrate. Confluent keratinocyte cultures were stained with either BM165 (*a*) or control media (*b*). An intense broad band of staining occurs at cell borders. The observed fluorescence correlates with intercellular space, as shown by low magnification TEM in sections taken through the cell layer parallel to the culture substate (*c*). If the cells are first removed with EDTA, the BM165 antibody directs homogeneous staining of the culture substrate (*d*). Bars: 20 nm.

IV collagen (Sakai et al., 1982), to laminin (see Materials and Methods), or to type VII collagen (Sakai et al., 1986*a*; not shown). It was also not seen when antibodies of the same immunological subtype, but of irrelevant specificity were used (not shown). The antigen is present upon the substrate underneath the cells as shown by strong fluorescence of the entire plastic substrate after removal of the cells with 10 mM EDTA (Fig. 3 *d*).

Electron-microscopic visualization of the antigen in keratinocyte cultures shows linear deposition of immunogold conjugates uniformly across the substrate upon a fine electron-dense feltwork (Fig. 4 *a*). The feltwork continued under the cell, but was often unlabeled. Thickenings could occasionally be seen along the keratinocyte plasma membrane that resembled immature hemidesmosomes (Fig. 4 *b*), similar to structures observed by others (Compton et al., 1989). The ultrastructural immunolocalization studies of BM165 in keratinocyte cultures were complicated by the rounding and detachment of the keratinocytes during long incubations with concentrated BM165 antibody. Scanning microscopy showing the altered morphology of the BM165 incubated cells compared to the morphology of untreated keratinocytes is shown in Fig. 4, *c* and *d*, respectively. Detached keratinocytes readily replated onto plastic and grew with equal vigor compared to untreated cells, indicating that the rounded and detached cells were not metabolically compromised by the antibody treatment (not shown).

To pursue these observations, the effects of the BM165 antibody on cell attachment and detachment were further investigated and quantitated (Fig. 5). As shown, the BM165 antibody inhibits, to >80%, keratinocyte attachment to both plastic and an endogenous keratinocyte matrix (Fig. 5, *A* and *B*). The antibody has no significant effect on fibroblast attachment to either of these matrices. In a similar fashion, the BM165 antibody also induces detachment of adherent keratinocytes without affecting fibroblasts (Fig. 5 *C*). Antibodies to laminin, type IV collagen, and type VII collagen could not duplicate these effects. It is interesting to note that while fibroblasts attach well to the keratinocyte matrix, the mechanism must differ from that used by keratinocytes.

The data shown in Fig. 3 *a* indicates that confluent keratinocyte cultures show no intracellular fluorescence. To evaluate the occurrence of substrate deposition of the antigen relative to time of plating, keratinocytes were plated at low density, and the development of fluorescence was observed as a function of increasing cell density. At 6 h after plating,

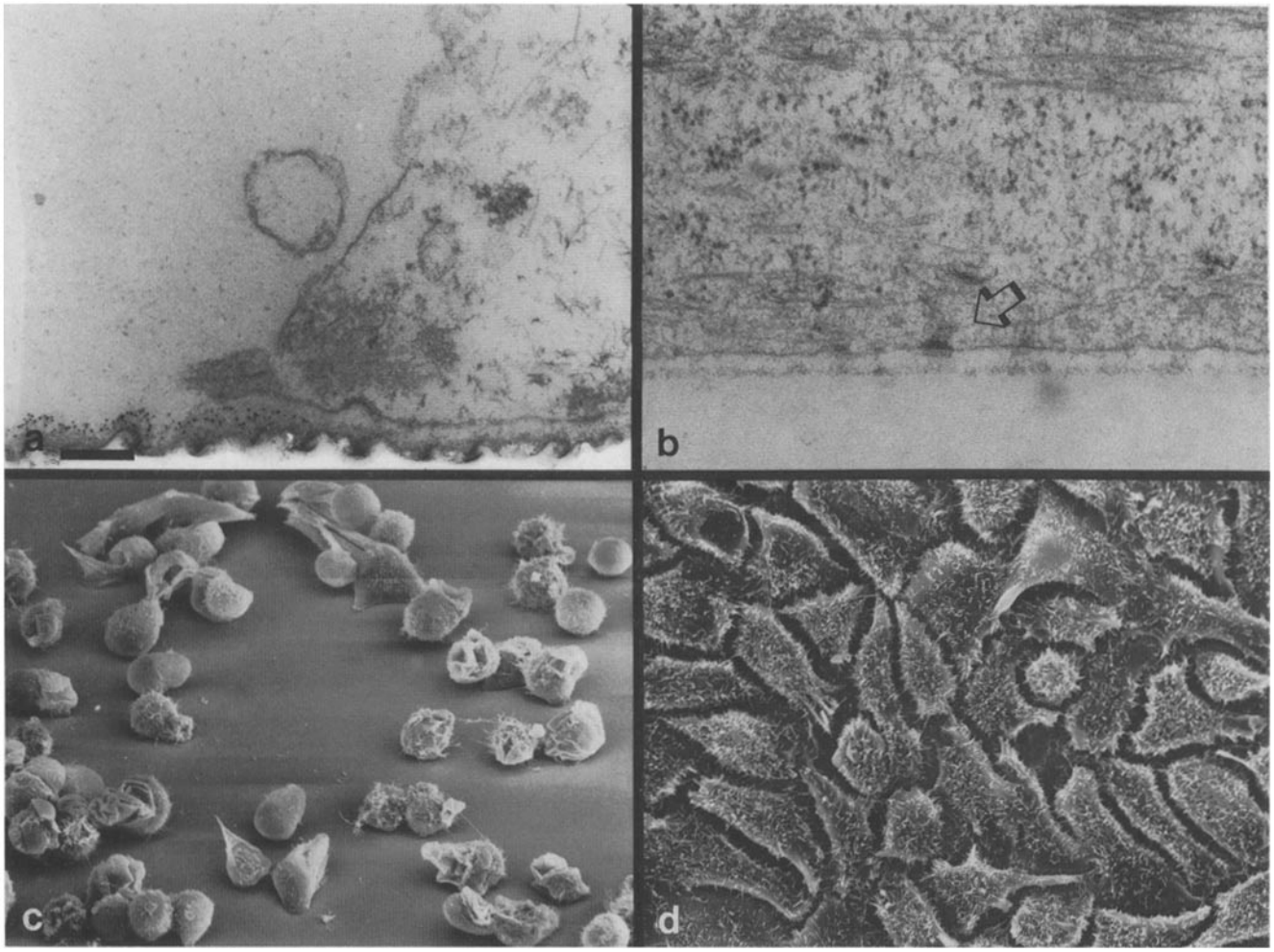

Figure 4. The BM165 antigen immunolocalizes along a continuous subcellular matrix in keratinocyte cell culture. Keratinocytes were grown to near confluency and either fixed immediately (*b* and *d*) or washed with PBS and incubated with BM165 mAb (50 μg/ml) followed by 5-nm gold–conjugated secondary antibody before fixation. For TEM, samples were embedded and sectioned perpendicular to the substrate. The antibody induced rounding of keratinocytes and localized to a feltlike matrix present on the culture substrate (*a*). In untreated keratinocytes a continuous matrix is observed under the cell (*b*). Structures resembling immature hemidesmosomes are often observed on the basal cell surface (*b*, *arrow*). The micrographs exhibit differences because of plastic (*a*) vs. glass (*b*) substrates. The morphology of rounded and untreated cells was also compared by SEM (*c* and *d*). Bars: *a* and *b*, 200 nm; *c* and *d*, 20 μm.

only intracellular fluorescence is observed (Fig. 6 *a*). By 24 h, individual cells and cell clusters can be seen showing both perinuclear intracellular fluorescence and fluorescent staining of the substrate immediately adjacent to the cells (Fig. 6, *b*, *d*, and *e*). In some cases, cells appear to have migrated, leaving behind fluorescent stain attached to the substrate (Fig. 6, *d* and *e*). As the cell clusters enlarge (Fig. 6 *c*), only the peripheral cells demonstrate intracellular fluorescence, suggesting that the cells at the interior of the clusters are no longer synthesizing this antigen. These results correlate with previous observations that cell growth and migration occur at the periphery of keratinocyte colonies and internal cells are quiescent (Barrandon and Green, 1987).

To partially characterize the antigen, the immunogen was fractionated from keratinocyte medium by immunoaffinity chromatography using the BM165 antibody, and was analyzed by polyacrylamide gel electrophoresis (Fig. 7). Before disulfide reduction, two species were visualized by staining with Coomassie blue (lane *1*). Both molecular species were immunoblot positive (lane *2*). The predominant species migrated with an ~400,000 M_r, and a minor species 440,000 M_r was often seen.

After disulfide bond reduction, four major electrophoretic species are resolved (lane *3*, *arrow*); 165,000, 155,000, 140,000, and 105,000 M_r. None of these bands are immunoreactive with polyclonal antiserum to EHS laminin, or with mAbs to human A, B1, or B2 chains (data not shown). Only the 165,000 species (and an immunoreactive smaller species that does not correspond to any of the chemically stained bands, and presumed to be a degradation product) contains the BM165 epitope as shown by Western blot (lane *4*). The disulfide-bonded 400- and 440-kD species were separately excised from the gel, reduced with 2-mercaptoethanol, and the reduction products were separated by electrophoresis. The 440-kD species contains the 165, 155, and 140-kD chains (lane 5) and a small amount of a 220-kD species seen only faintly by Commassie stain (lane *3*). This 200-

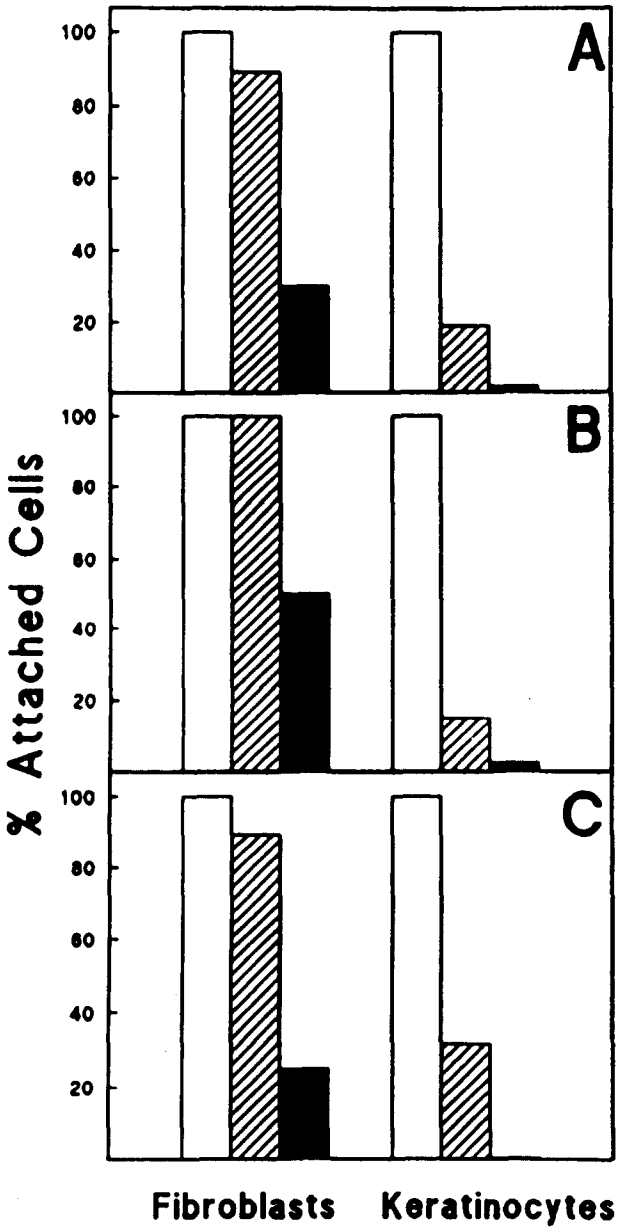

Figure 5. The BM165 antibody inhibits keratinocyte, but not fibroblast attachment. The effects of 50 μg/ml BM165 (▨) and 5 mM EDTA (■) on keratinocyte and fibroblast attachment were compared to untreated controls (□). (*A*) Attachment to plastic. (*B*) Attachment to endogenous keratinocyte matrix. (*C*) Detachment after 24 h of culture.

kD species also contains the BM165 epitope. The 400-kD species contains the 165, 140, and 105-kD chains (lane *7*). The results are consistent with identification of a molecule with three nonidentical chains. The difference in the electrophoretic migration of the nonreduced species can be explained by a conversion of the 155 to 105 kD by proteolysis. The results also suggest that the 165-kD chain is related to the 220-kD chain. Recently, a precursor product relationship between the 200 and the 165-kD chains was confirmed by biosynthetic pulse-chase experiments (M. P. Marinkovich, personal communication). It is not clear if these proteolytic events are physiological.

Rotary shadow imaging of the purified antigen shows a linear molecule containing a central rod of 107 nm (Fig. 8). The molecule is seen in two forms. The most common image appears as an extended dumbbell (Fig. 8, *b–e*). Often one knob appears smaller than the other. The least abundant is asymmetric with a large globule at one end and two smaller globules at the other (Fig. 8, *f* and *g*). The images are unlike any previously reported, to our knowledge. The relative abundance of the two images, and the additional knob on the larger species is consistent with the possibility that the larger image is contributed by the 440-kD molecule.

Discussion

We have isolated a protein from human keratinocyte culture that is involved in the attachment of the cell to plastic in vitro and to the basement membrane in vivo. The simplest model that explains the data is that the identified 165-, 155- and 140-kD chains form a single molecule with the characteristic rodlike morphology shown by rotary shadowing with two small globules at one end and a larger globule at the other end. A second molecule contains the 165, 140, and 105 kD is represented by the major rotary-shadowed image, lacking the second small globule at one end. These two molecules differ because of cleavage of the 155-kD chain to the 105-kD chain. At this time, we are unsure if this proteolytic event is physiological. We propose the name "kalinin" for this molecule, derived from the ancient Greek ΧΑΛΙΝΟΣ, meaning a bridle or a thong.

The immunolocalization of kalinin to human skin strongly suggests that this antigen is the ultrastructural element described as the anchoring filament. The rodlike shape and the length demonstrated by rotary shadowing of the antigen is also consistent with this assignment. The finding that the majority of the kalinin localizes to the lamina densa after antibody-induced rupture of the dermal–epidermal junction suggests that the BM165 antibody epitope lies near the region of the antigen responsible for binding to the hemidesmosome. The opposite end of the antigen appears to be buried in the lamina densa. The observed linear deposition of colloidal gold beneath the basal-dense plate suggests that this is the site of interaction with the hemidesmosome. When the contact between the hemidesmosome and the anchoring filament is broken by binding of the antibody, the antigen–antibody complex remains associated with the basement membrane.

Alternatively, the orientation of the molecular elements of the basement membrane zone seen by conventional microscopy may be entirely artifactual. Electron-miroscopic examination of rat incisor, tongue, and gingiva prepared by rapid freezing and freeze substitution demonstrate a homogeneous 25–100-nm thick electron-dense basement membrane completely lacking a lamina lucida (Goldberg and Escaig-Haye, 1986). The same result has been obtained with the dermal–epidermal junction of human skin in this laboratory (D. R. Keene, personal communication). Therefore, it is entirely possible that the lamina lucida is an artifact resulting from the cell shrinking away from the basement membrane, and the lamina densa is the residue of the entire basement membrane. If this is the case, it is likely that kalinin is entirely within the basement membrane, with only one end

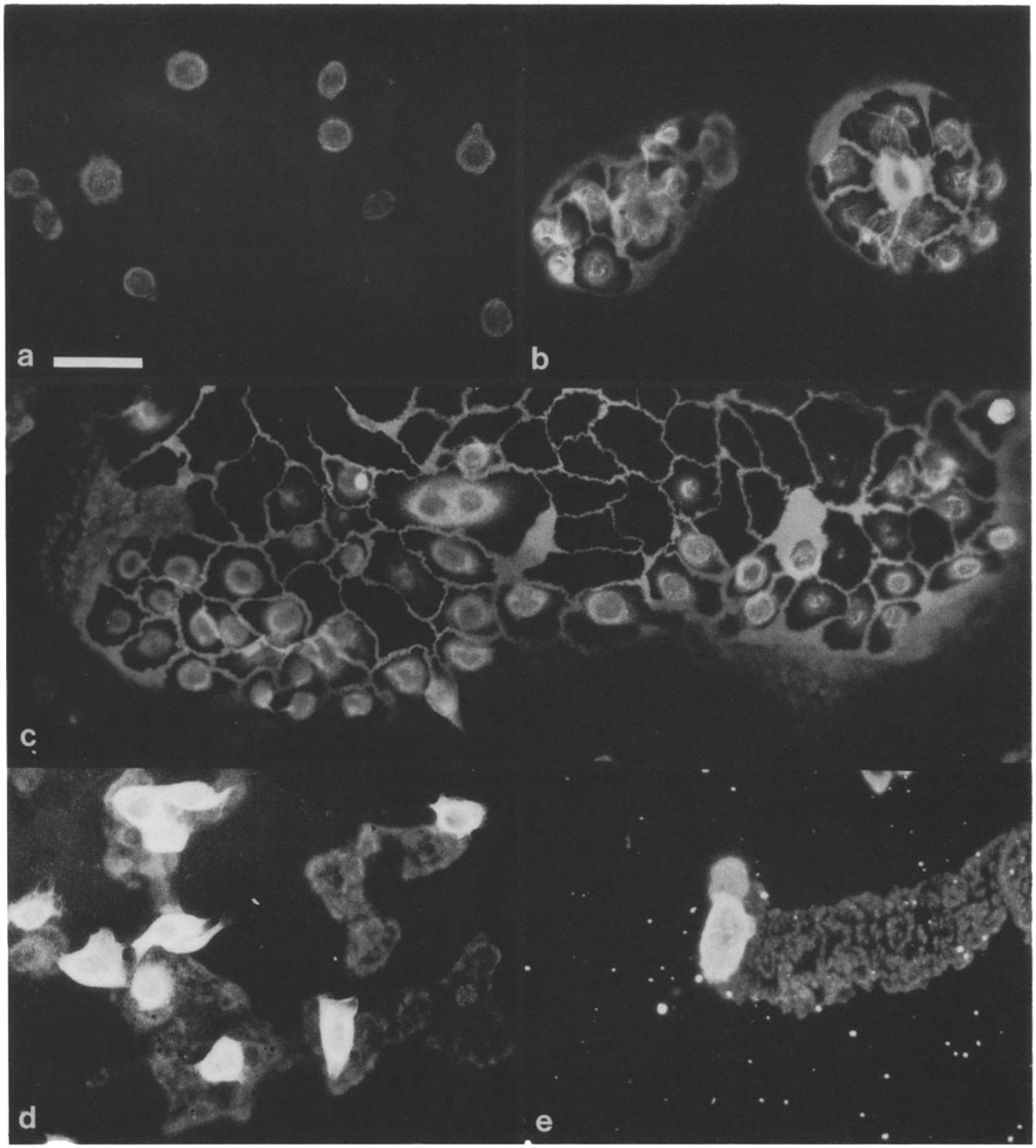

Figure 6. Localization and synthesis of the BM165 antigen correlates with growing and migrating cells. Keratinocytes were subcultured and at various times the BM165 antigen was localized by indirect immunofluorescence. After 6 h, attached cells exhibit intracellular fluorescence but have not yet flattened (*a*). At 24 h small cell clusters exhibit both intracellular and substrate labeling (*b*) and migrating cells have deposited the antigen along their paths (*d* and *e*). After 48 h the staining of larger clusters is heterogeneous (*c*). Interior cells exhibit only the matrix staining seen in confluent cultures, while peripheral cells also stain intracellularly. Bar, 50 μm.

concentrated at the site where the hemidesmosome contacts the basal lamina. The anchoring filaments would then reflect those species within the basal lamina that are strongly bound to the hemidesmosome and become taught and linear as they are pulled from the basement membrane as the cell shrinks away.

The data also suggest that in developing or regenerating epithelia, kalinin is initially distributed uniformly upon the

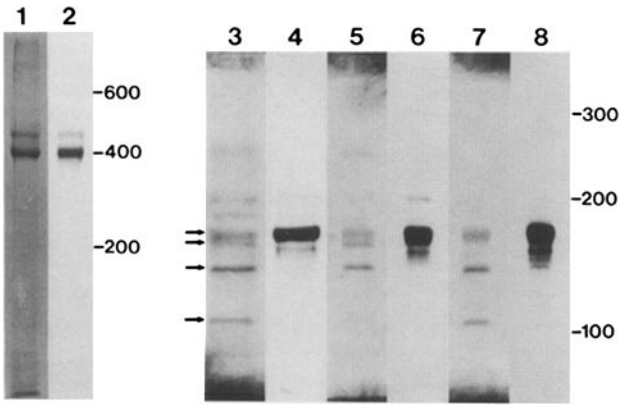

Figure 7. Electrophoretic analysis of the BM165 antigen isolated from keratinocyte culture medium. As described in Materials and Methods the antigen was affinity purified from spent keratinocyte culture medium. When analyzed by SDS-PAGE before reduction two species are detected by Coomassie blue staining, a major species migrating with a 400,000 M_r and a slower migrating minor component at 440,000 (lane *1*). The BM165 mAb identifies both bands upon western blot analysis (lane *2*). SDS-PAGE analysis of reduced material identifies four major bands after Coomassie blue staining; 165,000, 155,000, 140,000, and 105,000 M_r (*arrows*). Some minor, slower migrating components are also visible. Only the 165,000 is recognized by mAb BM165 (lane *4*). The nonreduced 440,000 and 400,000 were cut out and their reduction products evaluated separately. The 440,000 species gives reduction products with M_r of 165,000, 155,000 and 140,000 (lane *5*). The 400,000 species gives bands of 165,000, 140,000 and 105,000 M_r (lane *7*). In both cases only the 165,000 species is identified by mAb BM165 upon western blot analysis (lanes *6* and *8*). Sometimes a minor component of 200,000 M_r is also recognized by the BM165 mAb (lane *6*).

migration substrate, and becomes reorganized upon maturation of the attachment complex. The hypothesis is supported by the observation that keratinocytes cultured either on plastic or glass deposit kalinin uniformly upon the substrate, not solely beneath what appear in culture to be immature hemidesmosomes.

Rotary shadowing of kalinin indicates that it is an asymmetric molecule. This conformation is consistent with our hypothesis that one site on kalinin molecules is capable of interaction with receptors on the keratinocyte surface and another part remains buried within the lamina densa, thus providing cell–substrate adhesion. This impression is further supported by the observed disruption of cell–substrate contact upon incubation of cultured cells with the antibody, and the consistent and dramatic de-epithelization of skin caused by the BM165 antibody.

Kalinin appears to be synthesized only by growing keratinocytes. This observation suggests that if we are correct that kalinin is required for keratinocyte attachment to substrates, it may be important to consider the state of confluence of cells to be used for successful re-epithelialization of burn wounds. The results also suggest that kalinin may be deficient or altered in individuals with the blistering conditions such as junctional epidermolysis bullosa (Eady, 1987) or herpes gestationis (Katz and Provost, 1987).

The authors gratefully acknowledge the assistance of Dr. Clytie Rimberg in implementing the keratinocyte culture system used in these studies. The authors acknowledge the excellent technical assistance provided by Ms. Marie Spurgin and Ms. Catherine Ridgeway.

These studies were supported by U.S. Public Health Service grant AR35689 and the Shriners Hospital for Crippled Children, and used facilities partially provided by the R. Blaine Bramble Research Foundation and the Fred Meyer Charitable Trust.

Received for publication 21 December 1990 and in revised form 6 May 1991.

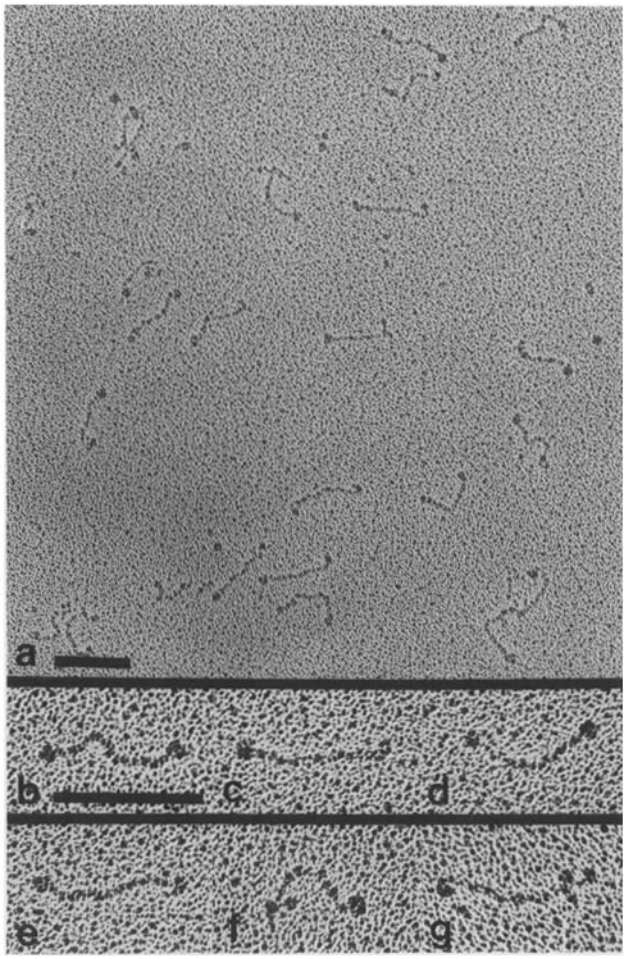

Figure 8. Rotary shadow analysis of the BM165 antigen. After affinity purification the sample was dialyzed against 0.2 N acetic acid and rotary shadowed using standard techniques. A low magnification field (*a*) and several representative molecules (*b–g*) are shown. The 107-nm-long molecules appear as extended rods with globular knobs at each terminus. Sometimes two knobs are resolved at one end (*f* and *g*). Bar, 100 nm.

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