

Kalinin Is More Efficient than Laminin in Promoting Adhesion of Primary Keratinocytes and Some Other Epithelial Cells and Has a Different Requirement for Integrin Receptors

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Abstract. Kalinin was purified from squamous cell carcinoma (SCC25) spent culture media using an immunoaffinity column prepared from the mAb BM165. The affinity-purified material was separated by SDS-PAGE into three bands of 165–155, 140, and 105 kD identical to those obtained from normal human keratinocyte cultures and previously identified as kalinin. Kalinin promoted adhesion of a large number of normal cells and established cell lines with an activity similar to other adhesion molecules such as the laminin–nidogen complex, fibronectin, or collagen IV. However, kalinin was a much better substrate than laminin–nidogen complex for adhesion of cells of epithelial origin including primary human keratinocytes. Adhesion to kalinin was followed by cell shape changes ranging from rounded to fully spread cells depending on the cell types. The adhesion-promoting ac-

tivity of kalinin was conformation dependent and was abolished by heat denaturation.

mAb BM165 prevented cell adhesion to kalinin but not to other extracellular matrix substrates. However, either complete or partial inhibition was observed with different cells suggesting the existence of at least two cell-binding sites on the kalinin molecule. Experiments inhibiting cell adhesion with function-blocking anti-integrin subunit antibodies indicated that both $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins are involved in the cellular interactions with kalinin, while for cell adhesion to classical mouse Engelbreth-Holm-Swarm laminin only $\alpha 6\beta 1$ integrins, and not $\alpha 3\beta 1$, appeared to be functional. Altogether, these results suggest that kalinin may fulfill additional functions than laminin, particularly for epithelial cells.

KALININ is a specific component of basement membranes that underly stratified epithelia. Using immunoelectron microscopy, it was previously localized to the anchoring filaments of the skin (Rousselle et al., 1991). The anchoring filaments are proposed to play a crucial role in the maintenance of the mechanical strength and cohesion of the epidermal–dermal junction, since in some forms of inherited blistering disease a split between epidermis and dermis occurs at the level of these filaments (Haber et al., 1985).

Kalinin has been identified and characterized in keratinocyte cultures as a disulfide-linked heterotrimer precursor with subunits of 200, 155, and 140 kD which is rapidly processed by cleavage of the 200- and 155-kD chains into 165- and 105-kD polypeptides, respectively (Rousselle et al., 1991; Marinkovich et al., 1992a). The three chains are folded into a rod with globular domains at both ends (Rousselle et al., 1991). Further immunological studies and partial cDNA sequencing have shown that kalinin is similar or even

identical to molecules discovered in other laboratories and named BM600/nicein (Verrando et al., 1987, 1993) or epiligrin (Carter et al., 1991; Domloge-Hultsch et al., 1992). Furthermore, these studies have demonstrated that kalinin/nicein/epiligrin represents a unique laminin isoform of smaller size than the original laminin isolated from the Engelbreth-Holm-Swarm (EHS) tumor (Timpl, 1989). One of the kalinin chains corresponds to the recently sequenced truncated B chain, B2t, a 130-kD variant of the laminin B2 chain but lacking several of the corresponding amino-terminal domains (Kallunki et al., 1992).

The family of laminin molecules therefore consists of an increasing number of members. Initially, laminin was identified and characterized in the basement membrane-producing and transplantable EHS tumor of mice as a cross-shaped heterotrimer with one heavy chain of 400 kD, the A chain, and two different light chains of ~ 200 kD, the B1 and B2 chains (Timpl et al., 1979). Later, several laminin chain variants were identified as cDNA clones and/or as polypep-

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1. *Abbreviations used in this paper:* EHS, Engelbreth-Holm-Swarm; NHK, normal human keratinocytes.

tides, the results indicating that different molecular isoforms of intact laminin exist. Smaller-size laminin A chain variants of 350–380, 230, and 190 kD have been described for heart muscle and Schwann cells (Paulsson and Saladin, 1989; Ehrig et al., 1990; Paulsson et al., 1991), endothelial cells (Tokida et al., 1990), and epithelial cells (Marinkovich et al., 1992b), respectively, and shown to associate with the classical B1 and B2 chains. Several B chain variants have also been identified: S-laminin, a 180–190-kD isoform of the B1 chain (Hunter et al., 1989) and the truncated B2t mentioned above (Kallunki et al., 1992).

The classical B1 and B2 chains have a widespread distribution in all basement membranes (Timpl, 1989), while expression of other chain variants varies with tissue localization. The isoform of the A chain of 350–380 kD, known as merosin, and the B1 chain homolog, or S-laminin, are, respectively, present largely in basal lamina of muscle and motor neuron synapses, respectively, but not in the basement membrane underlying epithelia (Engvall et al., 1990; Sanes et al., 1990). In contrast, the strongest mRNA signals for B2t chain are restricted to specific epithelial cells in skin, lung, and kidney (Kallunki et al., 1992), in agreement with the immunolocalization of kalinin/epiligrin/nicein to basement membranes underlying epithelia (Verrando et al., 1987; Carter et al., 1991; Rousselle et al., 1991). This also corresponds to the localization of K-laminin, an isoform that is assembled from a 190-kD A chain and classical B1 and B2 chains. Under some circumstances K-laminin is associated with kalinin (Marinkovich et al., 1992b).

The tissue-specific localization of variants suggests that different laminin isoforms could fulfill different functions. The major biological effects of isolated EHS laminin are: (a) to promote adhesion of a large number of neuronal and non-neuronal cells (Aumailley et al., 1987; Goodman et al., 1987; Dillner et al., 1988); and (b) to induce a wide array of cellular responses such as neurite outgrowth from neurons (Baron von Evercooren et al., 1982; Edgar et al., 1984), myoblast locomotion (Goodman et al., 1989), and epithelial polarization (Klein et al., 1988). However, for keratinocytes, laminin was found to inhibit their migration (Woodley et al., 1988) and to be a poor substrate for adhesion (DeLuca et al., 1990), although these cells directly abut a basement membrane *in vivo*. The diverse cellular responses are largely mediated by interactions with integrins (Ruoslahti, 1991; Hynes, 1992) and specific peptide sequences or conformation-dependent domains of the laminin molecule (Aumailley et al., 1990a,b; Deutzmann et al., 1990; Goodman et al., 1991). A major cell-binding site is located toward the end of the long arm of the molecule and is recognized by the $\alpha 6\beta 1$ integrin (Aumailley et al., 1987; Goodman et al., 1987; Sonnenberg et al., 1990) or in few cells by $\alpha 7\beta 1$ integrin (von der Mark et al., 1991). A minor cell-binding site has been mapped to the two most carboxy-terminal globules of the A chain (Sonnenberg et al., 1990; Sorokin et al., 1992). Further cellular interactions occur with the center of the cross formed by the short arms and are mediated by $\alpha 1\beta 1$ integrin for native laminin (Goodman et al., 1991; Hall et al., 1990), and by RGD-dependent integrins after partial proteolytic cleavage of the molecule (Nurcombe et al., 1989; Aumailley et al., 1990a).

It is difficult to purify intact laminin variants directly from tissues and information on their biological activities is still scarce. Merosin was found to induce neurite outgrowth and

to promote adhesion of different cell types in a manner similar to laminin (Engvall et al., 1992). An indication that kalinin could serve as an adhesion substrate comes from the observation that one anti-kalinin antibody, mAb BM165, induced rounding up of cultured keratinocytes (Rousselle et al., 1991). In addition, epiligrin or epiligrin-containing extracellular matrices deposited by normal or transformed epidermal cells were shown to be preferential ligands for $\alpha 3\beta 1$ integrin on keratinocytes (Carter et al., 1991), T lymphocytes (Wayner et al., 1993), and $\alpha 3$ -transfected erythroleukemia cells (Weitzman et al., 1993).

We have now purified kalinin from cell culture spent media by immuno-affinity chromatography and have been able to investigate in detail its ability to induce cell adhesion. Here, we report that kalinin is a very potent substrate for adhesion of a large variety of cells, being a much better epithelial cell adhesion substrate than laminin. Cell adhesion to kalinin is conformation dependent and involves both $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins.

Materials and Methods

Cell Cultures

Squamous cell carcinoma line SCC25 (CRL 1628; American Type Culture Collection) was grown in 50% Ham's F-12 and 50% DME (GIBCO BRL, Cergy Pontoise, France) supplemented with 10% fetal calf serum, 2 mM glutamine, hydrocortisone (0.4 μ g/ml), and a cocktail of antibiotics. Normal human keratinocytes (NHK) were isolated from neonatal foreskin (Boyce and Ham, 1985) and used either directly in cell adhesion assays (see below) or to initiate monolayer cultures in synthetic serum-free medium (Keratinocyte-SFM; GIBCO BRL). Spent culture media were regularly harvested from confluent NHK and SCC25 cells. After clarification and addition of protease inhibitors (5 mM EDTA, 50 μ M each PMSF and *N*-ethylmaleimide), the collected media were kept frozen at -20°C until further use for kalinin purification.

Human lung fibroblasts (L132) and hepatocytes (Chang) from ATCC and mouse myoblasts C2C12 were kindly provided by Dr. Ko (Institute for Microbiology, Cologne, Germany) and Dr. H. von der Mark (Institute for Experimental Medicine, Erlangen, Germany), respectively. All other cells listed in Table I have been previously described (Aumailley and Timpl, 1986; Aumailley et al., 1987, 1989). Cells were cultured in DME supplemented with 2 mM glutamine, a cocktail of antibiotics, and 10% fetal calf serum. For screening of kalinin-producing cells, the monolayers were incubated for 24 h in serum-free medium and the supernatants were harvested and kept frozen at -20°C until further use. When not otherwise mentioned cell culture reagents were from Seromed/Biochrom (ATGC, Moisy-Le-Grand, France).

Kalinin Purification

Immunoglobulin G1 fraction of mAb BM165 (Rousselle et al., 1991) was coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Pharmacia Biotech, St.-Quentin Yvelines, France) according to manufacturer's instructions. NHK or SCC25 spent media (500 ml) was passed sequentially over 25 ml of gelatin-Sepharose (Pharmacia Fine Chemicals, Pharmacia Biotech) and 10 ml BM165-Sepharose, both equilibrated in PBS. First affinity chromatography on gelatin-Sepharose was necessary and sufficient to remove fibronectin from the conditioned media as checked by immunoblotting with a polyclonal antiserum against fibronectin (not shown). Material bound to BM165-Sepharose was eluted using 1 M acetic acid and analyzed by SDS-PAGE and immunoblotting. The fractions containing kalinin were pooled, neutralized by dialysis against PBS, and kept frozen at -20°C . Protein concentration was determined according to Pierce (Pierce Interchim, Montluçon, France). Typical yields were 100–200 μ g of kalinin per 500 ml of conditioned medium.

Other Cell Adhesion Proteins

Laminin–nidogen complex was from the EHS tumor of the mouse (Paulsson

Table 1. Detection of Kalinin in Culture Media of Different Normal Cells and Established Cell Lines by Western Blotting with mAb BM165

Cells	Reaction with mAb BM165
Human origin	
Primary basal keratinocytes, NHK	+
Ovarian carcinoma, OVCAR-4	+
Mammary epithelia, HBL-100	+
Skin squamous carcinoma I, SCI I	+
Skin squamous carcinoma II, SCI II	+
Mammary carcinoma, MCF-7	-
Mammary carcinoma, T47D	+
Hepatocytes, Chang	-
Skin epithelioid, A431	+
Fibrosarcoma, HT1080	-
Melanoma, A375	-
Rhabdomyosarcoma, A204	ND
Tongue squamous carcinoma, SCC25	+
Lung fibroblasts, L132	-
Other species	
Rat schwannoma, RN22	-
Mouse myoblasts, C2C12	-
Rat parietal yolk sac, Pys-2	ND
Rat glioma, RuGli	-
Mouse melanoma, B16F10	-

1 ml of cell culture-conditioned media was precipitated by ammonium sulfate (33% saturation) and loaded onto 5% acrylamide gels under reducing conditions. Resolved polypeptide bands were transferred to nitrocellulose filters and blotted against mAb BM165. The samples reacting with mAb BM165 are marked "+" and nonreacting samples are marked "-". ND, not determined.

et al., 1987) and kindly donated by Dr. R. Timpl (Max-Planck Institute, Martinsried, Germany). Human plasma fibronectin was either purchased (GIBCO BRL) or kindly provided by S. Bégué (Centre de Transfusion Sanguine, Lyon, France). Human collagen IV was from human placenta (Tiollier et al., 1990) and kindly provided by Dr. J. Tiollier (Imedex, Chaponost, France).

Cell Adhesion and Inhibition Assays

Multiwell tissue culture plates (96 wells; Costar Corp., Dutscher, France) were coated with serial dilutions of kalinin, laminin-nidogen complex, collagen IV, or fibronectin (0–40 µg/ml, 100 µl/well) by overnight adsorption at 4°C. In some assays kalinin was heat denatured for 10 min at 70°C or 85°C before being used for coating the wells. After saturation of the wells with 1% BSA (fraction V; Sigma Chimie, Saint-Quentin-Fallavier, France) the plates were immediately used for cell adhesion assays in serum-free medium as detailed previously (Aumailley et al., 1989). The extent of adhesion was determined after fixation of the adherent cells with 1% glutaraldehyde in PBS, staining with 0.1% crystal violet and the color read with an ELISA reader (MR5000; Dynatech, Guernsey Channel Islands, UK) at 570 nm. A blank value corresponding to BSA-coated wells (<5% of maximal cell adhesion) was automatically subtracted. Adherent cells were photographed using a phase contrast microscope equipped with camera (Olympus Corp., Lake Success, NY).

For cell adhesion inhibition experiments with anti-substrate antibodies, the coated wells were incubated with serial dilutions in PBS of either mAb BM165 or antiserum against laminin for 60 min at room temperature as indicated in the corresponding figures. In inhibition assays with anti-integrin antibodies, suspended cells were mixed with dilutions of antibodies before plating onto the coated wells.

Each assay point was derived from triplicate wells.

Antibodies and Peptides

mAb BM165 (Rousselle et al., 1991) was purified from ascites by affinity chromatography on Protein G Sepharose (Pharmacia Fine Chemicals, Pharmacia Biotech). Polyclonal antisera raised in rabbits against purified laminin-nidogen complex, fibronectin, or collagen IV were kindly provided by Dr. R. Timpl (Max-Planck Institute, Martinsried, Germany). Purified mAb

GoH3 against integrin α6 subunit was kindly donated by Dr. A. Sonnenberg (The Netherlands Cancer Institute, Amsterdam, The Netherlands). The following mAbs against integrin subunits were from commercial sources (Tebios Pharmaceuticals, Inc., San Diego, CA, and GIBCO BRL): P4C10 against β1, 3E1 against β4, and PIB5 against α3. Synthetic RGDS peptide was from Sigma Chimie).

Gel Electrophoresis and Immunoblotting

SDS-PAGE was carried out according to Laemmli using 5% or 3 to 5% gradient acrylamide gels. Proteins were either stained with Coomassie Brilliant Blue or transferred to nitrocellulose filters (Bio Rad Laboratories, Ivry, France) for Western blotting according standard procedures. Low molecular weight markers were from Pharmacia Fine Chemicals, Pharmacia Biotech.

Results

Cell culture-conditioned media from different cell lines were screened by Western blotting with mAb BM165 which recognized the 165-kD band of kalinin (Rousselle et al., 1991). Out of 17 different cell lines or strains only 8 showed a positive reaction with mAb BM165 (Table I). The 165-kD band was particularly intense for the culture medium of the tongue squamous cell carcinoma line SCC25 which was then chosen for further purification of kalinin by affinity chromatography on BM165-Sepharose 4B. The material retained on the column was eluted with 1 M acetic acid and the peak fractions were analyzed by SDS-PAGE. The material was resolved into three major bands which could be stained by Coomassie Brilliant Blue and migrated at 165–155, 140, and 105 kD (Fig. 1 A). No other bands could be seen throughout all the fractions corresponding to the peak, except in the heaviest loaded lanes of the gel (lanes 2 and 3) where traces of two slower migrating bands at ~200 kD accounted for less than 5% as judged by intensity of staining. This material is likely to correspond to K-laminin (Marinkovich et al., 1992b) and was not further investigated. The SDS-PAGE patterns of the material immuno-purified from SCC25 or normal human keratinocyte culture media were identical under reducing and nonreducing conditions and migrated with different mobilities than fibronectin and laminin-nidogen complex used as molecular weights markers (Fig. 1 B). The chromatographic and electrophoretic patterns were reproducible and observed with more than 10 preparations. In addition, the 165-kD band was recognized after Western blotting by mAb BM165 as previously reported for kalinin prepared from NHK (Rousselle et al., 1991). None of the three major bands in the kalinin preparation were blotted with anti-laminin antibodies (Fig. 1 C) or antibodies against nidogen, collagen IV, or fibronectin (not shown). Based on these results it was concluded that SCC25 cells were suitable for kalinin preparation with the advantage of being a continuous cell line, cheaper, and easier to maintain in culture than normal human keratinocytes. The material eluting in fractions corresponding to lanes 1–9 (Fig. 1 A) was pooled and used to investigate biological activity of kalinin.

Kalinin was tested for its ability to induce adhesion of normal cells and of different cell lines in comparison to laminin-nidogen complex, collagen IV, and fibronectin. As shown for epithelial mammary cells HBL100 and subcultured NHK, kalinin was as good an adhesion substrate as laminin-nidogen, collagen IV, or fibronectin, and the adhesion plateaus were roughly the same for all four substrates (Fig. 2, A and D). Maximum adhesion was obtained at con-

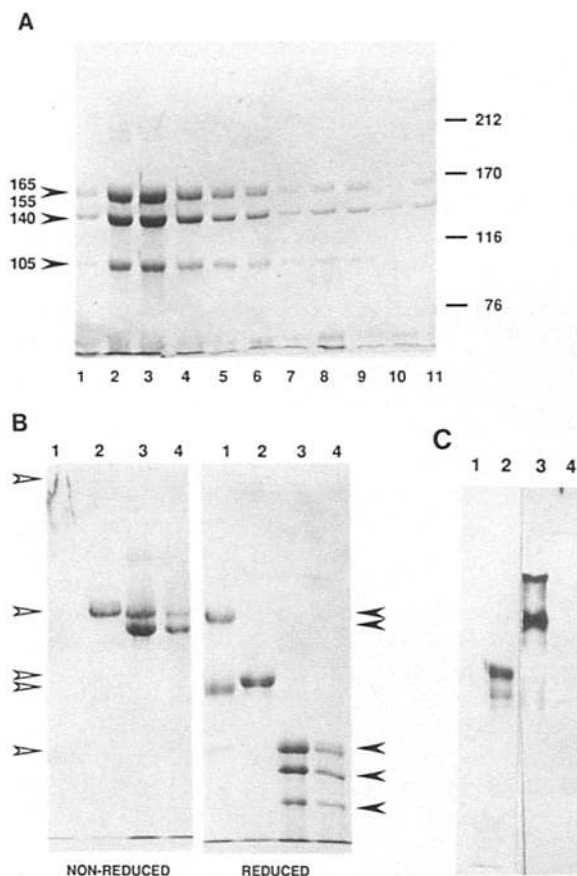


Figure 1. SDS-PAGE and western blot analysis of immuno-affinity purified kalinin from cell culture media. (A) SCC25 cell culture medium was affinity-chromatographed on BM165-Sepharose and 50- μ l aliquots of eluted fractions (1 ml) corresponding to the absorbance peak (lanes 1–11) were ethanol-precipitated and resolved by SDS-PAGE on a 5% acrylamide gel under reducing conditions. Protein bands were stained with Coomassie Brilliant Blue. Migration of molecular weight markers are shown at the right of the gel. Arrowheads to the left of the gel indicate the position of the polypeptide bands corresponding to kalinin. (B) SCC25 cells and NHK culture media were affinity-chromatographed on BM165-Sepharose and the eluted material (50- μ l aliquots) were analyzed by SDS-PAGE on 3–5% gradient gels under reducing and nonreducing conditions. (Lanes 1 and 2) Laminin–nidogen complex (1 μ g) or fibronectin (2 μ g), respectively, used as molecular weight markers. (Lanes 3 and 4) Material purified from SCC25 and NHK culture medium, respectively. Open arrowheads indicate the migration of laminin–nidogen complex and fibronectin. Closed arrowheads denote the migration of kalinin trimers (partially and fully processed forms of 440 and 400 kD, respectively) under nonreducing conditions, and of kalinin chains (165–155, 140, and 105 kD) after reduction. (C) Western blot analysis of BM165-Sepharose affinity purified SCC25 culture media. The reduced samples were run on a 5% acrylamide gel followed by transfer to nitrocellulose filters. (Lanes 2 and 4) SCC25 kalinin (0.1 μ g/lane); (lanes 1 and 3) laminin–nidogen complex (0.1 μ g/lane). Lanes 1 and 2 were blotted with mAb BM165 and lanes 3 and 4 with a polyclonal antiserum against laminin.

concentrations of the same order of magnitude for all substrates which were 0.5 and 5 μ g/ml of kalinin for subcultured NHK and HBL100 cells, respectively. In contrast, kalinin was a much better adhesion substrate than laminin for freshly iso-

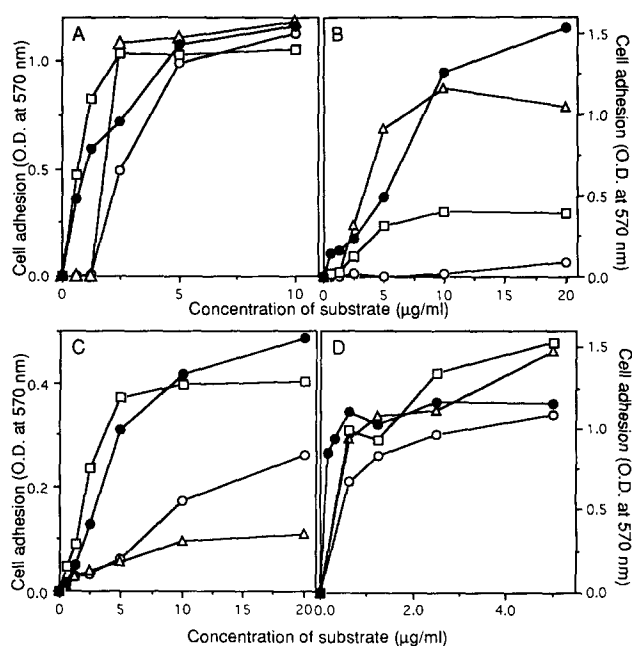


Figure 2. Dose-dependent cell adhesion to kalinin in comparison to laminin–nidogen complex, fibronectin, and collagen IV. Multiwell plates were coated with different concentrations of kalinin (●), laminin–nidogen complex (○), collagen IV (□), and fibronectin (Δ) as indicated on the figure. Cell adhesion was measured after 30 min using a colorimetric reaction as described in Materials and Methods. Each point represents the average of triplicate wells. Representative experiments for (A) HBL100 cells, (B) T47D cells; (C) freshly isolated normal human keratinocytes; (D) normal human keratinocytes at passage 3.

lated NHK or epithelial carcinoma cell line T47D both in terms of substrate concentration and maximal adhesion (Fig. 2, B and C).

The adhesion-promoting activity of kalinin was further tested and compared to other adhesion substrates for a large number of cells. For many of the tested cells, all four substrates induced rapid cell adhesion which reached the same plateau level as that observed with laminin–nidogen. However, for several cell lines of epithelial origin, kalinin was much better than laminin–nidogen (Fig. 3). Particularly, and in addition to the T47D cells and the primary keratinocytes shown in Fig. 2, B and C, plateau levels of adhesion to kalinin were found to be high also for OVCAR-4, Pys-2, MCF-7, and SCl II cells while they were low on laminin–nidogen (Fig. 3).

Adhesion to kalinin was accompanied by spreading of the cells in a manner comparable to that observed for other adhesion molecules as shown for HBL100 cells (Fig. 4). For all other cells, adhesion to kalinin was accompanied by shape changes, however, cell morphology was variable ranging from round to fully spread depending on the type of cells as it is illustrated with selected examples (Fig. 5). In addition, cells adhering to kalinin exhibited a tendency to associate laterally and isolated single cells were rarely observed (Fig. 5).

Cell adhesion-promoting activity of kalinin was conformation dependent as shown by loss of activity upon heat denatu-

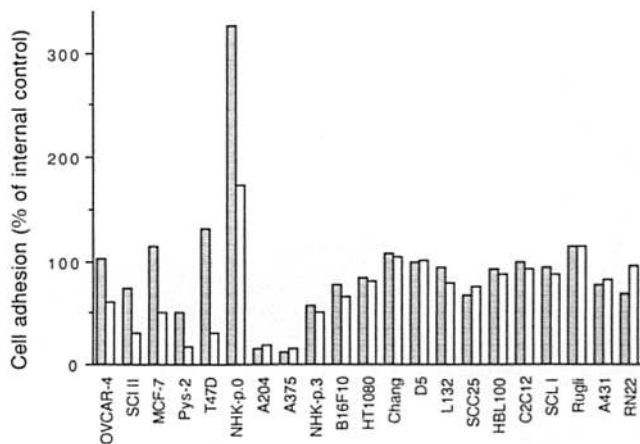


Figure 3. Adhesion efficiency of kalinin for normal keratinocytes and different cell lines in comparison to other cell adhesion molecules. Dose-response curves were established for all the different cell lines following the same protocol as for Fig. 2. For comparison of substrate efficiency, cell adhesion to fibronectin was chosen as an internal control and was arbitrarily set as 100% for each experiment. Maximum of adhesion to kalinin (*gray columns*) and to laminin-nidogen complex (*white columns*) was then expressed in percent of cell adhesion to fibronectin. NHK-p.0 and NHK-p.3 denote normal human keratinocytes at passage 0 and passage 3, respectively.

ration of the molecule. After heating kalinin at 70°C, adhesion of HT1080 cells was abolished only partially and after heating at 85°C adhesion dropped below 1% of control (Fig. 6). The same dramatic loss of activity upon heat denaturation of kalinin was observed with most of the other cells and residual activity ranging between 10 and 20% of control was observed with SCC25, HBL100, C2C12, and RuGli cells (Table II, *right column*).

To assess the specificity of cell adhesion to kalinin, substrate coats of kalinin, laminin-nidogen complex, collagen IV, and fibronectin, were incubated with mAb BM165 prior to the assays. mAb BM165 totally prevented HT1080 cell adhesion to kalinin but not to the other substrates (Fig. 7 A). Conversely, anti-laminin antibodies had no effect on cell adhesion to kalinin while they prevented cell adhesion to laminin-nidogen complex (not shown). The effect of mAb BM165 on adhesion of other kalinin-adhering cells was analyzed and two patterns of inhibition were observed. Inhibition was dose dependent and either complete or partial as shown for NHK or RuGli cells, respectively (Fig. 7 B). The concentration of mAb BM165 leading to 50% inhibition of adhesion (IC_{50}) was determined for other cells (Table II). It was in the range of 0.04–4 $\mu\text{g/ml}$ for those cells which could be totally inhibited (as for HT1080 cells and NHK, Fig. 7, A and B) while it was in the range 8.5–>10 $\mu\text{g/ml}$ for cells

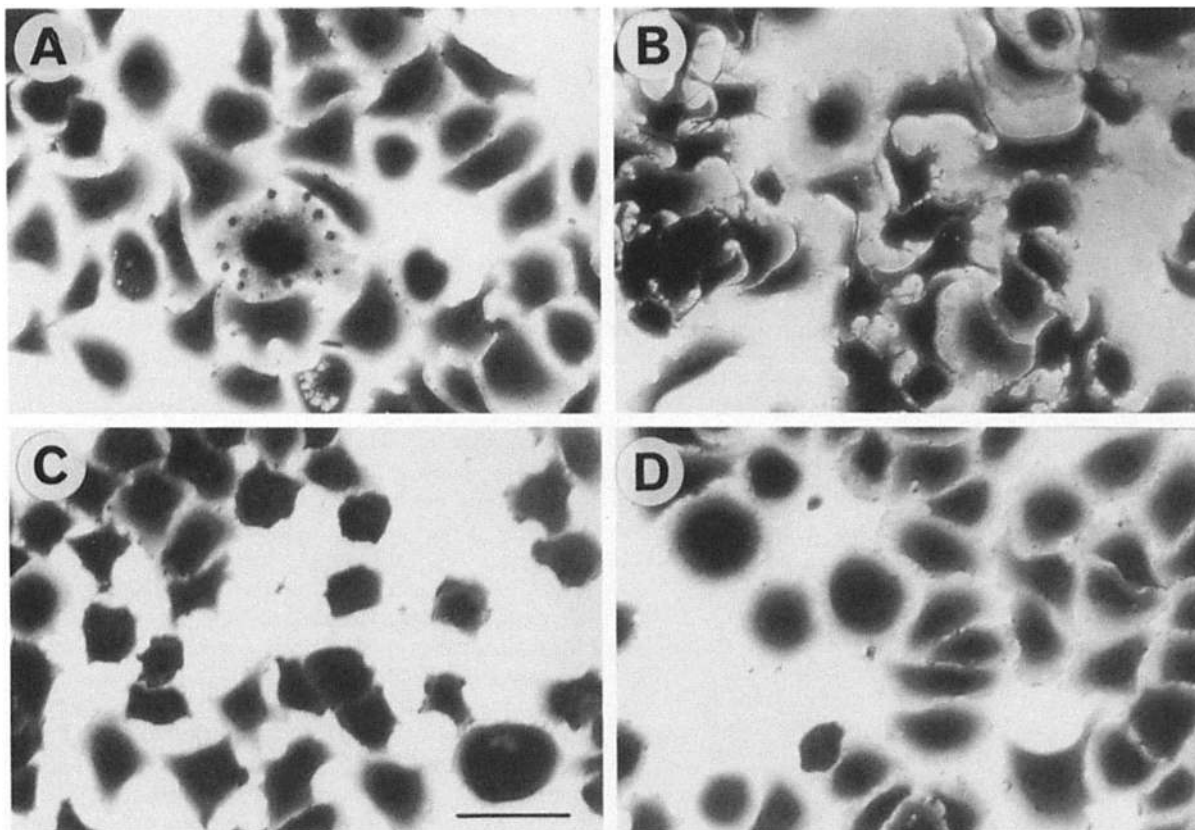


Figure 4. Comparison of spreading patterns of HBL100 cells on kalinin (A), laminin-nidogen complex (B), collagen IV (C), and fibronectin (D). Experiments were carried out as described in Fig. 2. After 30 min attached cells were fixed, stained, and photographs were taken under phase-contrast microscopy. Bar, 50 μm .

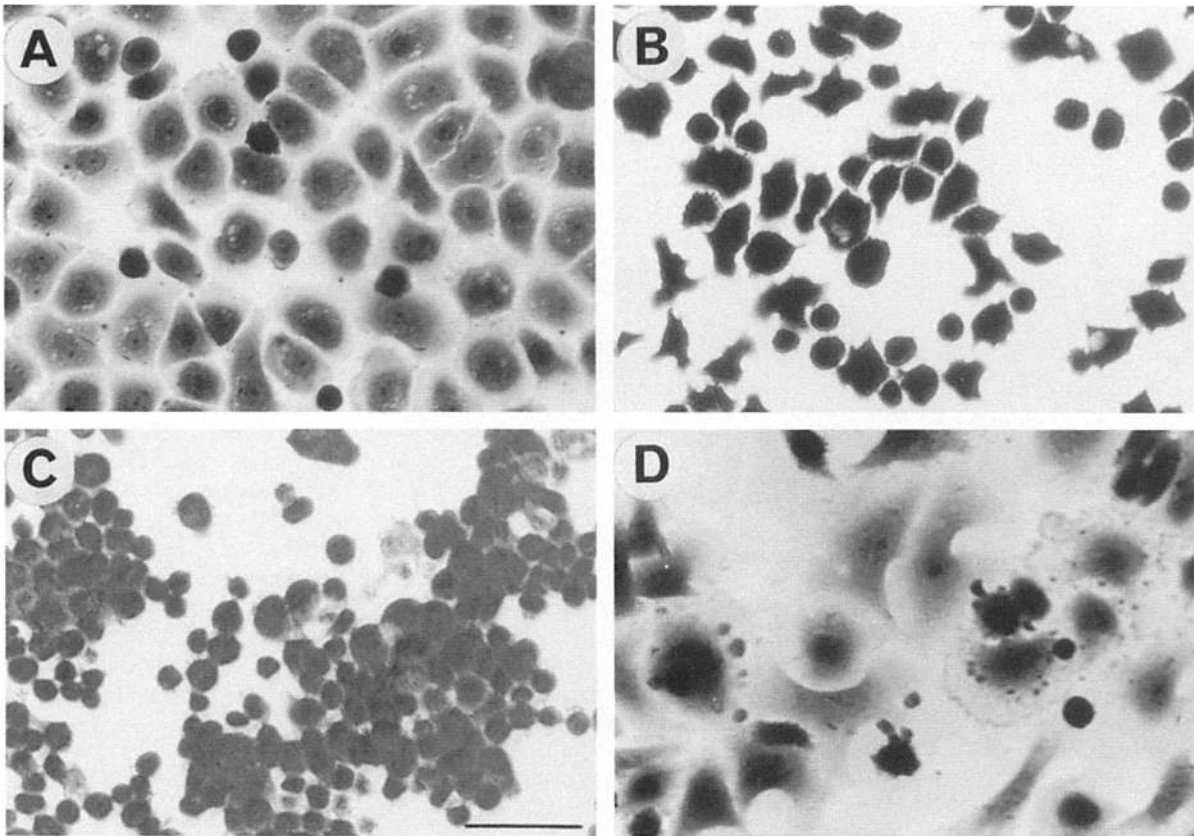


Figure 5. Different spreading patterns of cells seeded on kalinin. Experiments were performed as described in Fig. 2. Photographs were taken as for Fig. 4 after 30 min for NHK (A), Pys-2 (B), and OVCAR-4 (D) cells and 60 min for T47D (C) cells. Bar, 50 μ m.

which could be only partially inhibited like RuGli, C2C12, and HBL100 (Fig. 7 B and Table II) in the concentration range used, suggesting that kalinin could have several cell-binding sites and that BM165 was interfering with only one.

To get insight into the possible receptors involved in cell adhesion to kalinin, experiments were carried out with anti-integrin-specific antibodies. Adhesion of HBL100 cells to kalinin was substantially inhibited by mAb P4C10 against β 1 integrin subunit but not by mAb 3E1 against the β 4 subunit. Combining P4C10 and 3E1 did not result in a stronger inhibiting effect than each mAb used alone and similar results were observed for cell adhesion to laminin (Fig. 8). mAb GoH3 against α 6 integrin subunit was not inhibitory of cell adhesion to kalinin while mAb PIB5 against the α 3 subunit partially prevented adhesion. Combining GoH3 and PIB5

completely abrogated adhesion. In contrast, mAb GoH3, but not PIB5, inhibited cell adhesion to laminin and combination of both antibodies did not result in better inhibition than GoH3 alone. Cell adhesion to kalinin or to laminin was not affected by the peptide RGDS (Fig. 8). Identical experiments with anti-integrin antibodies and RGDS peptides carried out with OVCAR-4 (Fig. 8 B), SCC25, HT1080 and Scl II (not shown) cells produced the same results. Other cells were not tested.

Discussion

Basal lamina are known to be polymorphic in their morphology and functions and this is likely to be due in part to the composition of genetic variants in the basic constitutive molecules (Sanes et al., 1990). These variants appear to differ in their biological properties as we have shown here for cellular interactions with kalinin and laminin. Previous and indirect results have suggested that kalinin may be a substrate for the adhesion of keratinocytes (Rousselle et al., 1991). We have now immuno-purified kalinin from culture medium of SCC25 cells in amounts and level of purity suitable for a detailed investigation of its biological activity. Our studies show that kalinin has cell adhesion-promoting activity for a large variety of cell types, comparable in magnitude to other adhesive proteins but with distinct specificities. Although there are differences from one cell type to another, the coating concentrations of kalinin necessary to induce maximal

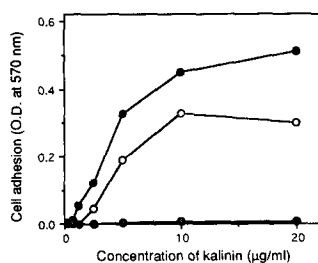


Figure 6. Effect of heat denaturation on cell adhesion-promoting activity of kalinin. Multiwell plates were coated with kalinin at the indicated concentrations without (●) or with prior heating at 70°C (○) or 85°C (⊙). HT1080 cells were used in the experiment shown and cell adhesion was determined as for Fig. 2.

Table II. Effects of mAb BM165 and Heat Denaturation on Cell Adhesion to Kalinin

Cells	IC ₅₀ (μg/ml) on substrates of		Cell adhesion activity of kalinin after heating at 85°C (% of control)
	Kalinin	Laminin-Nidogen	
MCF-7	<0.04	>10	1
SCC25	0.06	>10	15
NHK	0.09	>10	NT*
L132	0.15	>10	NT
OVCAR-4	0.21	>10	3
SCI II	0.21	>10	1
RN22	0.21	>10	2
B16F10	0.21	>10	1
T47D	0.40	NA‡	0
Pys-2	0.57	NA	NT
Chang	0.85	>10	NT
A431	1.17	>10	NT
HT1080	4.0	>10	1
HBL100	8.5 (57)§	>10	18
C2C12	>10 (18)	>10	10
RuGli	>10 (47)	>10	20

Inhibitory capacity (IC₅₀ = 50% inhibition) of mAb BM165 for cell adhesion to kalinin and laminin-nidogen complex was determined from dose-response curves as shown in Fig. 5. Cell adhesion to heated (85°C) and nonheated kalinin was measured as shown in Fig. 6 and the results are expressed as a percent of cell adhesion to nonheated kalinin set as 100%.

* NT, nontested.

‡ NA, nonapplicable since these cells have a too low adhesion to laminin-nidogen complex.

§ The values indicate the maximum of inhibition observed with 10 μg/ml of mAb BM165. For all the other cell types maximal inhibition was ≥90%.

adhesion are in a range similar to those for the laminin-nidogen complex, collagen IV, or fibronectin. For instance, roughly similar plateau levels were observed for subcultured NHK on coats of kalinin, laminin-nidogen, collagen IV, or fibronectin at 0.5–1 μg/ml while HBL100 cells required higher coating concentrations of 5 μg/ml. However, a distinct cellular specificity was observed since kalinin, but not

laminin-nidogen complex, induced adhesion of several cell types of epithelial origin, including freshly isolated human foreskin keratinocytes. In this context, it is worth noting that upon subcultivation NHK acquired adhesiveness to laminin and to fibronectin. A similar observation has been previously reported for fibronectin (Adams and Watt, 1991). Our data suggest that culture conditions might be very critical for

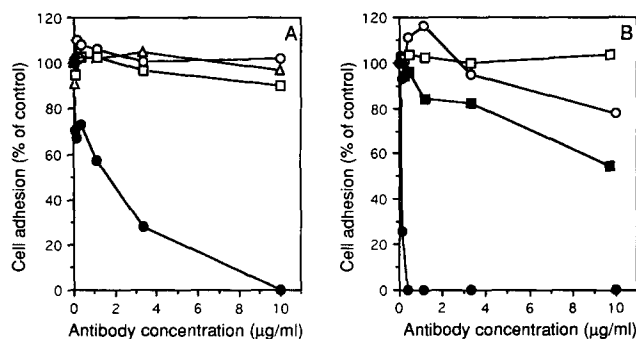


Figure 7. (A) Effect of mAb BM165 on adhesion of HT1080 cells to kalinin, laminin-nidogen complex, fibronectin, or collagen IV. Multiwell plates were coated with kalinin (●), laminin-nidogen complex (○), fibronectin (Δ), or collagen IV (□) at 10 μg/ml. After saturation with 1% BSA, the wells were incubated with the indicated concentrations of mAb B165 for 60 min before cell adhesion assays. Extent of cell adhesion was measured with a colorimetric reaction and expressed as the percent of adhesion in the absence of antibodies. (B) Inhibition of RuGli and NHK adhesion to kalinin by mAb BM165. Multiwell plates were coated with 10 μg/ml of kalinin (closed symbols) or laminin-nidogen complex (open symbols). Experiments were conducted as in A with RuGli cells (■, □) or NHK (●, ○).

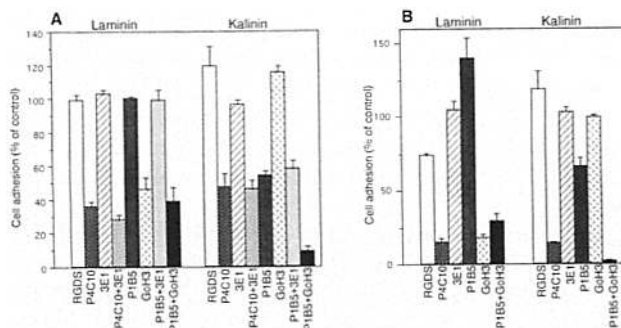


Figure 8. Effects of RGDS peptides and anti-integrin antibodies on HBL100 (A) and OVCAR-4 (B) cell adhesion to kalinin and laminin-nidogen complex. Multiwell plates were coated with kalinin or laminin-nidogen complex at 10 and 20 μg/ml, respectively. After saturation with 1% BSA, the cells were seeded in the presence of RGDS (0.5 mg/ml), or of mAbs P4C10 against β1 (1:1,000), 3E1 against β4 (1:50), GoH3 against α6 (6 μg/ml), PIB5 against α3 (1:100) used alone or in combination (same final concentrations as when used alone) as indicated under the columns. After 30 min, the extent of adhesion was determined as for previous figures. The results are expressed as a percent of the control without peptides or antibodies.

investigating the adhesiveness properties of normal epidermal cells.

Cell adhesion to kalinin was abrogated by heat denaturation at 85°C even for cells (RuGli) previously reported to bind heat-denatured laminin with good efficiency (Goodman et al., 1991). These observations suggest that conformational requirements for cell adhesion to kalinin and laminin are not strictly identical. First, it has been shown that the major cell adhesion site of laminin which is located towards the end of the long arm depends on secondary and tertiary structures and is destroyed by heating at 65°C or urea denaturation (Aumailley et al., 1987; Goodman et al., 1987; Deutzman et al., 1990). Here we showed that cell adhesion-promoting activity of kalinin is also conformation dependent, however, it is resistant to heating at higher temperature than that of laminin. Second, an additional heat insensitive cell-binding site has been demonstrated on the EHS laminin short arms (Goodman et al., 1991). For kalinin cell-binding activity was almost completely abrogated after heating suggesting that the latter site may be lacking in agreement with the observation that short arms are truncated on the molecule (Kallunki et al., 1992; Vailly et al., 1994).

However, we cannot exclude the possibility of several cell-binding sites on kalinin since inhibition of cell adhesion with mAb BM165 was either complete or partial depending on the investigated cells (Table II). We have previously made similar observations for the inhibition of cell adhesion to laminin with antibodies against the major cell-binding domain (Aumailley et al., 1990b, 1991; Sonnenberg et al., 1990; Goodman et al., 1991). Specifically, adhesion of OVCAR-4 or HT1080 cells, which solely depends on the major cell-binding site of laminin, was completely inhibited with antibodies, however, adhesion of HBL100 or RuGli cells, which are known to have interactions with an additional cell-binding site on the laminin short arms, was only marginally impaired. Here, we have shown that among other cells adhesion of OVCAR-4 and HT1080 cells to kalinin could be completely inhibited by mAb BM165 while that of HBL100 and RuGli cells was partially prevented. This parallelism suggests that on kalinin a single binding site could be available for cells like HT1080 and OVCAR-4 while at least two sites could be targets for RuGli and HBL100 cells. Direct proof for the existence of one or several cell-binding domains on kalinin will, however, necessitate their isolation which will be difficult given the small quantity of material that can be purified.

Cell adhesion to kalinin seems to involve receptors of the $\beta 1$ family as suggested by the inhibitory effects observed with mAb P4C10 directed against the integrin $\beta 1$ subunit. Cell adhesion to kalinin was, however, not affected by mAb GoH3 against $\alpha 6$ integrin subunit when used alone, while it was partially prevented by mAb P1B5 against $\alpha 3$ integrin subunit, and almost completely inhibited with a combination of both mAbs (Fig. 8). The observation of an inhibitory effect of P1B5 is in agreement with other reports showing that epiligrin or epiligrin-containing extracellular matrices are ligands for $\alpha 3\beta 1$ integrin (Carter et al., 1991; Wayner et al., 1993; Weitzman et al., 1993). Here, using functional assays with HBL100 and OVCAR-4 cells which possess, respectively, a large and a restricted repertoire of integrins (Sonnenberg et al., 1990), we have observed that antibodies against $\beta 1$ or against both $\alpha 3$ and $\alpha 6$ integrin subunits are

necessary and sufficient to abolish adhesion to kalinin. Lack of inhibition of cell adhesion to kalinin with mAb GoH3 alone could be explained by a compensation by $\alpha 3\beta 1$ integrins due to higher ligand affinity or receptor number. This is in contrast to cell adhesion to EHS laminin for which a major role was previously recognized for $\alpha 6\beta 1$ integrin and not for $\alpha 3\beta 1$ (Aumailley, 1990b; Hall et al., 1990; Sonnenberg et al., 1990). In fact, seen retrospectively, several reports indicate that cell adhesion and integrin binding might differ for laminin variants. A lack of inhibition by mAb GoH3 was observed for cell adhesion to preparations of human placental laminin (Brown and Goodman, 1991; Champliand, M.-F., and M. Aumailley, unpublished observations) which contains several isoforms, including merosin (Engvall et al., 1990) and kalinin (Rousselle et al., 1991). Using affinity chromatography of cell membrane extracts on human placenta laminin, it was found that $\alpha 3\beta 1$ integrin was primarily retained (Gehlsen et al., 1988) and that $\alpha 6\beta 1$ integrin could bind to the affinity column only after immunodepletion of the samples with anti- $\alpha 3$ antibodies. However, with similar experiments on mouse laminin binding of only $\alpha 6\beta 1$ integrin was observed (Sonnenberg et al., 1991b).

Extracellular matrix proteins are likely to transmit signals to cells via the integrins and to induce a wide array of cellular responses (Hynes, 1992). For $\alpha 6\beta 1$ integrin a role in signalling for epithelial cell polarization and differentiation has been demonstrated in kidney tubules (Sorokin et al., 1990). The cellular responses which could be induced via $\alpha 3\beta 1$ integrins are more elusive. Several reports have shown that $\alpha 3\beta 1$ integrins are located at cell-cell contact domains of epidermal cells and could therefore play a role in cell-cell communications (Kaufmann et al., 1989; Carter et al., 1990a,b; DeLuca et al., 1990; Larjava et al., 1990; Adams and Watt, 1991; Marchisio et al., 1991; Symington et al., 1993). Furthermore, anti- $\alpha 3$ antibodies increased keratinocyte mobility (Kim et al., 1992). Interactions of basal keratinocytes with basement membrane components are likely to be critical for tight adhesion of the cells as well as for maintenance of cell polarization and perhaps also for cell differentiation. Our results indicate that kalinin has the required biological properties to fulfill such functions in addition to its specific localization to basement membranes underlying epithelia. However, anchoring of the basal keratinocytes is not expected to be a lasting phenomenon since basal cells need to detach and move upwards to the upper layer of the epidermis as they undergo terminal differentiation. One could speculate that upwards movement of the basal keratinocytes could be induced by down-regulation of $\alpha 3\beta 1$ integrin. Preliminary results indicate a decrease of keratinocyte adhesion to kalinin with passaging, however, more extended investigations are still needed to support this hypothesis. Alternatively, a rearrangement of integrin subunits may occur during keratinocyte differentiation. In situ, it has been observed that $\alpha 6\beta 4$ and not $\alpha 6\beta 1$ integrins are present on the basal plasma membrane of keratinocytes (Carter et al., 1990a; Marchisio et al., 1991; Sonnenberg et al., 1991a). However, we did not get evidence that $\alpha 6\beta 4$ could be involved in the initial cellular recognition of kalinin or of laminin either. This integrin may have a role to play in latter events such as elaboration of stronger focal contacts with the basement membrane. Indeed, perturbation of such contacts with mAb 3E1 directed to the $\beta 4$ integrin subunit

has been observed several hours after plating epithelial cells (DeLuca et al., 1990; Ryyänen et al., 1991).

Kalinin is likely to be important also in the maintenance of the mechanical strength of the epidermal-dermal junction as suggested by several observations. Absence of immunoreactivity has been observed when GB3 serum (against BM600/nicein) was applied to skin sections of patients affected with a lethal blistering disease (Herlitz syndrome) where the split between dermis and epidermis occurs within the lamina lucida (Verrando et al., 1991). Patients affected with an acquired mucosal-predominant subepidermal blistering disease have circulating auto-antibodies reacting with epiligrin (Domloge-Hultsch et al., 1992). In addition when mAb BM165 was used to perform immunoelectron microscopy on skin fragments, extensive de-epithelization was observed as a consequence of cleavage within the lamina lucida (Rousselle et al., 1991). Our results suggest that the epitope recognized by mAb BM165 is located at the kalinin cell binding site or close to it since the antibody prevents cell adhesion to the protein. It is therefore reasonable to postulate that kalinin may be a target for pathological modifications leading to abnormal behavior of basal keratinocytes or to the disruption of the epidermal-dermal junction.

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