

Distribution of a 69-kD Laminin-binding Protein in Aortic and Microvascular Endothelial Cells: Modulation during Cell Attachment, Spreading, and Migration

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Abstract. Affinity chromatography and immunolocalization techniques were used to investigate the mechanism(s) by which endothelial cells interact with the basement membrane component laminin. Bovine aortic endothelial cell (BAEC) membranes were solubilized and incubated with a laminin-Sepharose affinity column. SDS-PAGE analysis of the eluted proteins identified a 69-kD band as the major binding protein, along with minor components migrating at 125, 110, 92, 85, 75, 55, and 30 kD. Polyclonal antibodies directed against a peptide sequence of the 69-kD laminin-binding protein isolated from human tumor cells identified this protein in BAEC lysates. In frozen sections, these polyclonal antibodies and monoclonal antibodies raised against human tumor 69-kD stained the endothelium of bovine aorta and the medial smooth muscle cells, but not surrounding connective tissue or elastin fibers. When nonpermeabilized BAEC were stained in an in vitro migration assay, there appeared to be apical patches of 69 kD staining in stationary cells. However, when released from contact inhibition, 69 kD was localized to ruffling membranes on cells at the migrating front. Permeabilized BAEC

stained for 69 kD diffusely, with a granular perinuclear distribution and in linear arrays throughout the cell. During migration a redistribution from diffuse to predominately linear arrays that co-distributed with actin microfilaments was noted in double-label experiments.

The 69-kD laminin-binding protein colocalized with actin filaments in permeabilized cultured microvascular endothelial cells in a continuous staining pattern at 6 h postplating which redistributed to punctate patches along the length of the filaments at confluence (96 h). In addition, 69 kD co-distribution with laminin could also be demonstrated in cultured subconfluent cells actively synthesizing matrix. Endothelial cells express a 69-kD laminin-binding protein that is membrane associated and appears to colocalize with actin microfilaments. The topological distribution of 69 kD and its cytoskeletal associations can be modulated by the cell during cell migration and growth suggesting that 69 kD may be a candidate for a membrane protein involved in signal transduction from extracellular matrix to cell via cytoskeletal connections.

ENDOTHELIAL cells are highly metabolic, polar cells that line blood vessels forming a nonthrombogenic surface (10, 18). In most vascular beds, endothelial cells synthesize and reside on a complex basement membrane composed of collagen types IV and V, heparan sulfate proteoglycans, glycoproteins such as laminin (Lm)¹, and other as yet unidentified components (27). Endothelial cells not only have to interact with components of the basement

membrane, but during the repair of soft tissue injury and during development endothelial cells must be able to interact with components of the interstitium such as collagen types I and III, which is critical for the survival of the organism. In vitro, individual purified matrix components have been shown to elicit unique biological responses from endothelial cells. A large body of data has been collected demonstrating the effects of the extracellular matrix (ECM) on the proliferation, migration, cell shape, and cytoskeletal organization of endothelial cells (25, 28, 29). However, the molecular mechanism(s) by which the endothelial cells interact with individual matrix components has yet to be elucidated.

Receptors/binding proteins for individual matrix components have been isolated from a variety of cell types. Surface-labeling studies have demonstrated that these binding proteins are cell surface associated (35, 39, 52) and liposome

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1. *Abbreviations used in this paper:* BAEC, bovine aortic endothelial cells; ECM, extracellular matrix; Fn, fibronectin; Lm, laminin; Lm-Seph, laminin-Sepharose CL-4B; PLP, paraformaldehyde-lysine-periodate; RFC, rat epididymal fat pad microvascular endothelial cells.

incorporation studies (35) as well as sequence analysis (44) have indicated that they are most likely integral membrane proteins. Some matrix binding proteins appear to have multiple binding specificities while others are mono-specific with the capacity for high affinity binding. For example, Integrin (cell surface adhesion molecule), a 140-kD complex of glycoproteins, was identified by monoclonal antibodies that perturb cell attachment (2, 9). Integrin is an example of a "promiscuous" binding protein in light of its ability to bind Lm as well as fibronectin Fn in an in vitro binding assay (16). This complex has been related to the 140-kD complex identified in mammalian cells that has been shown to bind immobilized Fn (35). In addition, a 47–48-kD collagen-binding protein that has been demonstrated in chicken fibroblasts (40), mouse parietal endoderm cells (20), and aortic endothelial cells (28; Yannariello-Brown, J., and J. A. Madri, manuscript in preparation) appears not to be specific for a particular collagen type but can bind to collagens type I, III, IV, and gelatin (20).

A cell surface binding protein for Lm with a molecular mass range between 65 and 72 kD has been reported in a variety of cell lines and tissues including tumor cells (1, 31, 36), skeletal muscle cells (22), and placenta (49). The 69-kD Lm-binding protein isolated from human breast carcinoma cells has been localized to the surface and the binding specificity of this molecule is restricted to Lm, binding with high affinity (1, 31, 36). Monoclonal (23) and polyclonal antibodies (Wewer, U., manuscript submitted for publication) have been generated to the 69-kD human Lm receptor isolated from breast carcinoma tissue. The monoclonal antibodies have been used to confirm the cell surface localization of the 69-kD protein (23) and in the molecular cloning of a cDNA (49).

It has been postulated that these cell surface ECM binding proteins/receptors are responsible for mediating cell attachment, spreading, and migration on the individual matrix components both in vitro and in vivo, and that these molecules may also be involved in matrix organization and signal transduction through cytoskeletal associations. The identification of cell surface molecules mediating interactions between endothelial cells and the subendothelial matrix would aid greatly in elucidating the mechanism(s) involved in endothelial cell-matrix interactions. This vital information will shed new light on mechanisms of endothelial cell migration during injury/repair, development, and angiogenesis.

In this communication we demonstrate the presence of binding proteins for the basement membrane component Lm using both immunolocalization and affinity chromatography on both large and microvascular endothelial cells. Polyclonal and monoclonal antibodies directed against a 69-kD Lm binding protein isolated from human breast carcinoma tissue were used to investigate the distribution of this molecule in situ and in vitro. We demonstrate that: (a) in the normal aorta and in capillary beds the antigen is found associated with the endothelial plasma membrane and with medial smooth muscle cells; (b) the in vitro distribution of the 69-kD protein is modulated during cell attachment, spreading, and during large vessel sheet migration; and (c) colocalization with actin microfilaments can be demonstrated in permeabilized cells as well as colocalization between the 69-kD protein and Lm.

Materials and Methods

Isolation and Culture of Various Cell Types

Bovine Aortic Endothelial Cells (BAEC). BAEC were harvested by the collagenase method as previously described (27) or by scraping. The protocol for the scrape preparation is as follows. Bovine aortae were obtained from the local abattoir sterilely by clamping the aorta with two hemostats before excision from the animal and immersed immediately into ice-cold PBS containing 10× penicillin/streptomycin and 5× fungizone. Excess adventitia was removed and the aorta splayed open to expose the endothelial layer. Red blood cells were washed away with a gentle stream of sterile PBS and the endothelial layer was gently scraped off using a No. 22 scalpel blade. The cells were washed in PBS, vortexed to break up cell sheets, resuspended in 5 ml of complete Dulbecco's minimal essential medium (cDMEM) and allowed to attach overnight in T-25cm² tissue culture flasks (Corning Glassworks, Medfield, MA). The following day the cell layers were washed to remove dead cells and fresh media was added. The cells were passaged and used for experimentation between passages 2–15. The endothelial cell nature of the cultures was confirmed by immunofluorescence staining for the presence of factor VIII-related antigen (Collaborative Research, Inc., Waltham, MA), angiotensin converting enzyme (generous gift of Dr. J. Roll, University of California, San Francisco), and Di-acetylated low density lipoprotein receptors (Miles Laboratories, Inc., Elkhart, IN). The morphological appearance and biological properties of the collagenase obtained cells were indistinguishable from the scraped cells.

Bovine Aortic Medial Smooth Muscle Cells. Bovine aortic smooth muscle cells were isolated, cultured, and characterized as previously described (12, 27).

Rat Epididymal Fat Pads. Microvascular endothelial cells were isolated from the epididymal fat pad (RFC) of male Sprague Dawley rats (kindly provided by Dr. James Boyer, Department of Medicine, Yale University) as previously described (26).

Preparation of Affinity Columns. Lm-Sepharose CL-4B (Lm-Seph) affinity columns were prepared from purified matrix components according to the method of Parikh et al. (33) as modified by Roll et al. (38) with the addition of 0.4 M NaCl to the cross-linking buffer. Briefly, purified Lm was exhaustively dialyzed into 0.2 M NaHCO₃, pH 9.0, plus 0.4 M NaCl and covalently cross-linked to CNBr-activated Sepharose. Unbound sites were blocked with 1% ethanolamine and the quenched columns were taken through two cycles of washes alternating between 0.1 M acetic acid plus 0.4 M NaCl and 1 M Tris-base pH 9.0 plus 0.4 M NaCl to remove all noncovalently bound components. Conditions were set so that between 1–2 mg of antigen was bound per ml of Sepharose. Control affinity columns included normal sheep IgG-Sepharose. Engelbreth-Holm-Swarm type IV collagen-Sepharose and cyanogen bromide-activated Sepharose quenched with ethanolamine.

BAEC Membrane Preparation. BAEC were grown to confluency on tissue culture grade 471 cm² trays (Nunc, Roskilde, Denmark). The cell layers were washed in cold TBS, pH 8.0, then scraped into the same buffer containing 5 mM EGTA plus 1 mM diisopropyl fluorophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM *N*-ethylmaleimide and 2 trypsin-inhibitory units aprotinin/100 ml (protease inhibitor cocktail). The cellular material was sedimented by centrifugation at 1,200 rpm for 7 min at 4°C. 5 vol of hypotonic buffer containing 10 mM Tris-HCl pH 8.0, 5 mM EGTA, 5 mM MgCl₂, and protease inhibitor cocktail were added to the cell pellet and incubated for 15 min on ice before disrupting with a Dounce homogenizer (Vineland, NJ) fitted with an A pestle. Sucrose was added to the buffer to achieve a final concentration of 0.25 M and the nuclei were removed by differential centrifugation (800 g for 2–3 min). The membrane containing supernatants were pooled then centrifuged at 20,000 g for 60 min at 5°C. Membranes were stored in protease inhibitor cocktail in 10 mM Tris, pH 8.0, with 20% glycerol at –70°C for up to 6 mo.

Affinity Chromatography. Cells membranes were solubilized in 3 vol 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40 plus protease inhibitor cocktail with stirring for 30 min in an ice bath. The suspension was then spun for 60 min at 100,000 g at 5°C to remove insoluble material. The supernatant was removed and the pellet resolubilized as described above. The NP-40 was diluted to 0.1% and the protein concentration adjusted to 300–500 µg/ml and then added to 5 ml of Lm-Seph (or control columns) which had been washed three times in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40. The columns were incubated with end over end mixing overnight at 4°C. At the end of the incubation period the columns were washed four times with 10 vol of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40

to remove unbound material. The columns were eluted with 6 M urea containing 50 mM Tris, pH 8.0, then dialyzed into 100 mM ammonium acetate, and concentrated by lyophilization. The samples were analyzed by standard 10% Laemmli SDS-PAGE under reducing conditions (21).

Immunoblotting BAEC Lysates with Polyclonal Anti-LB69. Immunoblotting BAEC lysates immobilized onto nitrocellulose filters was accomplished as previously described for breast carcinoma cell lysates. Briefly, BAEC were scraped into solubilization buffer containing 0.5% NP-40 and protease inhibitors and clarified by spinning for 10 min at 4°C at 10,000 g. The solubilized material was subjected to electrophoresis on 7.4% SDS-PAGE, transferred to nitrocellulose, and the filter was incubated in a blocking solution containing 5% nonfat dry milk and 0.2% antifoam B (A5757B; Sigma Chemical Co., St. Louis, MO) in TBS pH 7.4. Filters were then reacted with the KIRI-6 polyclonal anti-human 69 kD followed by an affinity-purified goat anti-rabbit Fc alkaline phosphatase (Promega Biotech, Madison, WI) at 0.9 µg/ml. The filters were stained with nitroterazolium (grade III; Sigma Chemical Co.) and 5-bromo-4-chloro-3-indolyl-phosphate (Sigma Chemical Co.).

Coating Bacteriological Plastic Dishes with Matrix Components. Bacteriological plastic dishes and tissue culture plastic dishes were coated with isolated purified matrix components in carbonate buffer, pH 9.3, as previously described (26, 29). We had determined previously that this protocol allows for reproducible, saturable, and native binding of the matrix components used in these studies (12, 26, 29).

Polyclonal and Monoclonal Antibody Production. Production and characterization of the rabbit anti-mouse Lm affinity-purified antibodies, the polyclonal rabbit and mouse monoclonal (IgM) anti-human 69-kD antibodies have been previously described (1, 23, 30). The ascities form of the monoclonal LR2 antibodies were used after being precipitated with 50% ammonium sulfate, then dialyzed into PBS pH 7.2.

Immunofluorescence Analysis of Frozen Tissue

Bovine Aorta. Bovine aortae were excised within minutes of death, washed in PBS, and immersed in paraformaldehyde-lysine-periodate (PLP) as described by McLean and Nikane (32). The aortae were then cut into 1–2 mm pieces and further fixed on ice for 4 h. Tissue pieces were embedded in O.C.T. embedding medium (Miles Scientific Div., Miles Laboratories) and frozen at –20°C. Frozen sections (6 µM) were prepared, postfixed in –20°C acetone for 2–3 min onto gelatin-coated slides, then quenched in PBS + 3% BSA overnight at 4°C. All antibodies were used at dilutions previously determined to be optimal. A rabbit anti-mouse IgM (µ chain specific) was used as a secondary antibody to identify the LR2 monoclonal antibodies followed by a goat anti-rabbit rhodamine-conjugated tertiary antibody. A goat anti-rabbit rhodamine-conjugated secondary antibody (Cappel Laboratories, Inc., Cochranville, PA) was used to identify the laminin receptor polyclonal antibodies. Secondary and tertiary antibodies were preabsorbed with live endothelial cells before use to eliminate nonspecific background binding. Controls for nonspecific staining included “normal” mouse ascities (prepared from mice injected intraperitoneally with the hybrid cell line SP2/0), normal mouse sera, and normal rabbit sera, none of which displayed any reactivity towards the sections tested. A positive monoclonal IgM control that was used was an anti-phosphotidyl choline monoclonal IgM, kindly provided by Dr. Kim Bottomly, Department of Pathology, Yale University. All antibodies were diluted in PBS + 1% BSA + 0.02% NaN₃, and all washes were performed in the same buffer. Coverslips were mounted in polyvinyl alcohol in glycerol and allowed to set overnight at 4°C. Photomicrographs were obtained using Kodak Ektachrome ASA 400 color slide film in an Olympus camera mounted onto a Zeiss microscope equipped for epifluorescence. Black and white prints were generated from XPI negatives duplicated from Ektachrome originals. Staining patterns and relative intensities of LB69 in the following studies were identical when either the polyclonal or monoclonal antibodies were used.

Rat Epididymal Fat Pads. Epididymal fat pads were isolated from male Sprague-Dawley rats and gently minced then either (a) mixed with O.C.T. embedding medium for sectioning or (b) incompletely digesting with collagenase to yield single cells before mixing with the O.C.T. Frozen sections (6 µM) were prepared and stained with antibodies as described for aortic sections.

Immunofluorescence Localization in Permeabilized Tissue Culture Cells at the Light Level. Immunofluorescence staining of tissue culture cells was performed as follows. Cultured cells were grown under the conditions specified by each experiment. The cell layers were washed three times

with PBS then fixed in either 3.5% paraformaldehyde in PBS prepared fresh from EM grade flakes or with PLP. In either case the cells were fixed on ice and quenched overnight in PBS containing 5% BSA, pH 7.2, at 4°C. When permeabilized cells were required the cell layers were fixed first, then permeabilized by incubating for 20 min in PBS containing 0.2% Triton X-100 at 4°C. Antibodies were added in a manner similar to that described in the previous section. In the case of double-label experiments, the appropriate controls were performed in which the LR2 monoclonals were incubated with the secondary for the anti-Lm antibodies, i.e., goat anti-rabbit Ig. The anti-Lm antibodies were incubated with the secondary for the LR2 antibodies, i.e., goat anti-mouse (µ chain specific) to eliminate the possibility of cross-reactivity between the primary antibodies and the inappropriate secondary antibodies. Controls were carried out in a manner similar to that described in the previous section.

Effects of Anti-69-kD Laminin-binding Protein on BAEC Attachment to Laminin. BAEC were grown to confluence on tissue culture plastic, harvested by trypsinization, washed in PBS, and allowed to regenerate their surface proteins by incubating in DMEM + 5% BSA for 1 h with gentle stirring. The cells were adjusted to a concentration of 20,000 cells/ml in PBS containing Ca⁺⁺ and Mg⁺⁺ in which 1:10 dilutions of either preimmune sera (normal rabbit serum), polyclonal, or monoclonal anti-69-kD antibodies had been made. The cells were preincubated on ice for 15 min before adding to 24 well cluster nontissue culture grade plastic dishes (Costar, Cambridge, MA) that had been precoated with 25.0 µg laminin/ml of carbonate buffer, pH 9.3 (12), then passivated with heat-inactivated BSA. BAEC were added in 1-ml vol and allowed to incubate for 1 h. At the end of the incubation period the cells were washed three times in 1 ml PBS to remove unattached cells. To determine the percent attached cells an enzymatic assay was used in which the levels of acid phosphatase were measured (8). Briefly, 200 µl of buffer containing 0.1 M sodium acetate (pH 5.5), 0.1% Triton X-100, and 10 mM *p*-nitrophenyl phosphate (Sigma Chemical Co.) were added per well. The cluster dishes were placed in a 37°C incubator for 2 h. The reaction was stopped with the addition of 20 µl of 1 N NaOH and the yellow color was monitored at 405 nM. Standard curves of attached BAEC were generated with each assay in order to determine cell number.

Effects of RGDS and YIGSR Synthetic Peptides on BAEC Attachment to Laminin. RGDS tetrapeptide was purchased from Peninsula Laboratories, Inc., Belmont, CA and used without further purification. GRGESP peptide was a generous gift of Dr. E. Rouslahti, La Jolla Cancer Research Institute, La Jolla, CA. YIGSR peptide was a generous gift of Dr. Yoshi Yamada, National Institute of Dental Research, National Institutes of Health, Bethesda, MD. The peptides were solubilized in PBS containing Ca⁺⁺ and Mg⁺⁺ at a concentration of 1.0 mM. BAEC, prepared as described above, were adjusted to a concentration of 60,000 cells/ml in PBS containing Ca⁺⁺ and Mg⁺⁺ and 1.0 ml of cells were added to 1.0 ml of peptide. The mixtures were then added to laminin-coated 35-mm-diam bacteriological plastic petri dishes (Falcon Labware, Oxnard, CA) (12). After a 1-h incubation the plates were washed three times with PBS, fixed with 100% methanol for 10 min, and stained with Hematoxylin and Eosin (Anatech, Battle Creek, MI). Numbers of attached cells were determined from counting six 0.03 mm² fields. Percent attachment was determined by comparing treated and untreated cells. Attachment was also assessed using the alkaline phosphatase assay as described above and both methods gave identical results.

Matrix Isolation and Purification

Fibronectin. Fn was purified as previously described with minor modifications (30). Specifically, the gelatin-eluted fractions were pooled and neutralized with 1 M Tris-base and further purified on a Sepharose S-200 column with the Fn eluting in the void volume.

Laminin. Laminin was isolated from the Engelbreth-Holm-Swarm tumor grown subcutaneously in lathyritic mice as previously described by Yurchenco et al. (45, 54).

Type IV Collagen. Type IV collagen was isolated from the lathyritic Engelbreth-Holm-Swarm tumor as described by Yurchenco and Furthmayr (53).

Types I and III Collagen. Type I and III collagen were isolated from calf skin and lung and human placenta as previously described (24).

Gelatin. Gelatin was prepared by heat denaturation of purified type I collagen as previously described (30).

The purity of all matrix components was determined by SDS-PAGE analysis and ELISA assay using affinity-purified antibodies as previously described (24, 26).

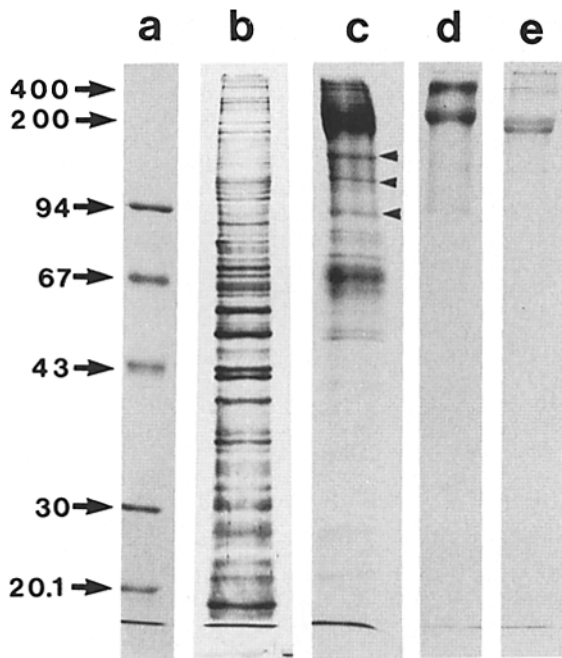


Figure 1. LB69 isolated from solubilized BAEC membranes using Lm-Seph affinity chromatography. BAEC membranes were isolated according to the methods described. The solubilized material was incubated with the affinity matrix, washed, and eluted with 6 M urea in 50 mM Tris, pH 7.2, with 5 mM EDTA. Eluted material was analyzed by 10% SDS-PAGE run in the presence of 2.5% β -mercaptoethanol. BAEC membranes were run in lane *b*; lane *c* is the eluted material from the Lm affinity column, including the major species at 69 kD; lanes *d* and *e* are Lm and type IV collagen standards, respectively. (Lane *a*) Molecular mass standards (Pharmacia, Inc., Piscataway, NJ) included phosphorylase b (94 kD), BSA (67 kD), catalase (60 kD), ovalbumin (43 kD), carbonic anhydrase (36 kD), anhydrase (30 kD), trypsin inhibitor (20.1 kD), ferritin (18.5 kD), and α -lactalbumin (14.4 kD).

Results

Identification of Lm-binding Proteins

BAEC membranes were solubilized in NP-40-containing buffer as described in the methods and incubated with Lm-Seph. Fig. 1, lane *c* shows the Coomassie Blue-stained Lm binding proteins eluted from the column. A major species eluted is a 69-kD band, which we will refer to as LB69 (Lm binding 69-kD protein). There are also a number of other bands seen on the gel, notably a series of bands that migrate with mobilities of 92, 110, and 125 kD (Fig. 1, *arrowheads*) under reducing conditions. Lower molecular mass bands seen migrating at 55 and 30 kD may represent proteolytic products or possibly other unique Lm binding proteins. We also see bands in the higher molecular mass regions of the gel (≥ 200 kD) which we interpret as ECM molecules, specifically Lm and IV, as determined by their mobilities on the gels (Fig. 1, *d* and *e*), immunoblotting with antibodies to laminin and type IV collagen (data not shown), and previous studies demonstrating Lm-Lm, IV-IV, and Lm-IV interactions (12, 53, 54). Elution of rabbit IgG-Sepharose and activated Sepharose quenched with ethanolamine columns revealed no specific bands retained by these columns (data

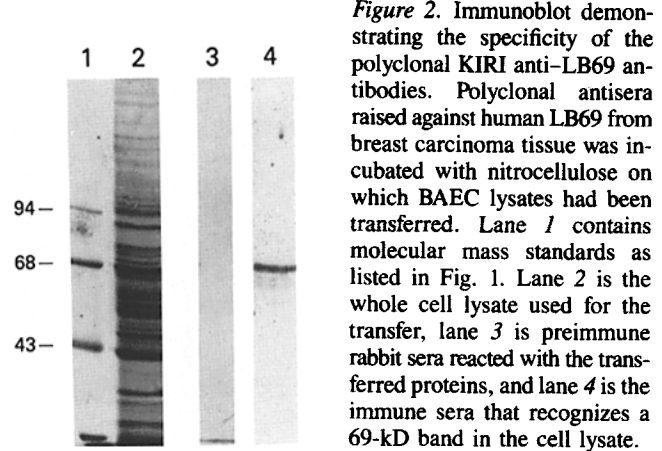


Figure 2. Immunoblot demonstrating the specificity of the polyclonal KIRI anti-LB69 antibodies. Polyclonal antisera raised against human LB69 from breast carcinoma tissue was incubated with nitrocellulose on which BAEC lysates had been transferred. Lane 1 contains molecular mass standards as listed in Fig. 1. Lane 2 is the whole cell lysate used for the transfer, lane 3 is preimmune rabbit sera reacted with the transferred proteins, and lane 4 is the immune sera that recognizes a 69-kD band in the cell lysate.

not shown). Elution of type IV collagen-Sepharose columns revealed a major band of 48 kD and a series of bands with mobilities of 92, 110, and 125 kD. (Yannariello-Brown, J., and J. A. Madri, manuscript submitted for publication).

Immunoblotting BAEC Lysates with a Polyclonal Antibody Raised against LB69

To determine if the antibodies raised against the human laminin receptor are recognizing a similar 69-kD protein species in BAEC, lysates were transferred to nitrocellulose and probed with a polyclonal antisera. A single 69-kD band is visualized as seen in Fig. 2, lane 4. Attempts to identify LB69 in transferred BAEC lysates with the monoclonal LR2 antibodies were unsuccessful even though the monoclonal antibodies recognize a 69-kD band in immunoblots of human platelets (data not shown). This is most likely due to species differences and the possibility that the epitope recognized by the monoclonal antibody is conformation specific and is sensitive to denaturation on nitrocellulose.

Effects of Antilaminin Receptor Antibodies, RGDS, and YIGSR Peptides on BAEC Attachment

When BAEC were incubated with polyclonal or monoclonal antibodies raised against the laminin receptor cell attachment to laminin-coated dishes was partially inhibited ($20 \pm 5\%$). Similar inhibition profiles were obtained when the synthetic peptide YIGSR was incubated with BAEC before plating (Basson, C. T., and J. A. Madri, manuscript in preparation). In contrast, when BAEC were incubated with the tetrapeptide RGDS before plating on laminin-coated dishes cell attachment was inhibited to a greater degree ($70 \pm 5\%$). Incubation of the cells with the synthetic peptide GRGESP elicited no inhibition of attachment in the concentration range tested.

Immunofluorescence Localization of LB69 In Situ

Before we investigated the *in vitro* localization/modulation of LB69 in BAEC and RFC we wanted to verify the presence of the antigen *in vivo*. When cross sections of bovine aorta were immunostained with the monoclonal LR2 antibodies, LB69 localized to the endothelium in a cortical fashion (Fig. 3, *B* and *D*, see *small arrows*). In areas where the endothelium had been lost during processing (Fig. 3, note *open*

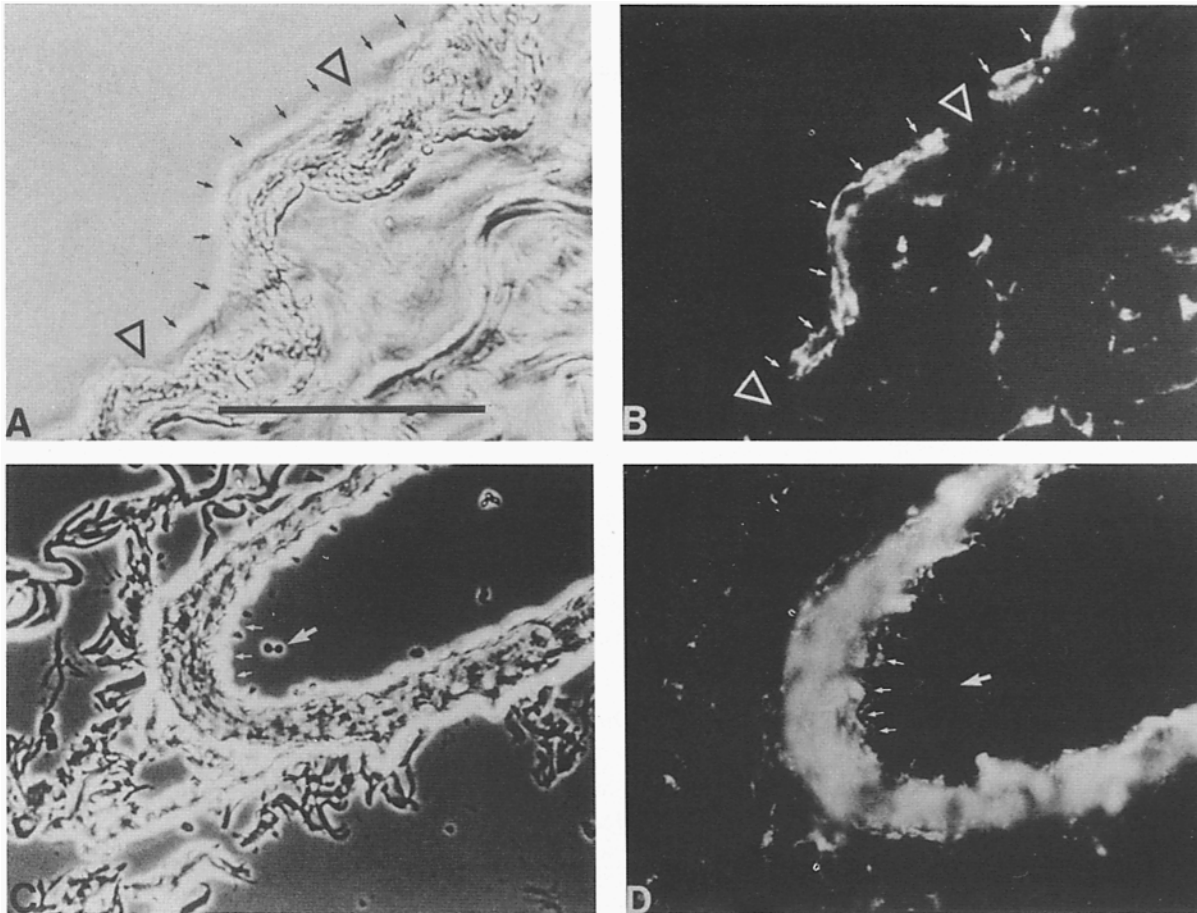


Figure 3. Immunofluorescence localization of the LB69 antigen in 6- μ m frozen sections of paraformaldehyde-fixed bovine aortic and arteriole tissue. Cross section of bovine aorta in a phase micrograph (*A*) and the identical section stained for LB69 using the monoclonal LR2 antibodies (*B*). Small arrows indicate areas on the vessel that have a continuous endothelium. Breaks in the monolayer are indicated by open triangles. (*C*) A bovine arteriole as seen in cross section by phase contrast microscopy and (*D*) stained with the monoclonal LR2 antibodies. The monolayer of endothelial cells is indicated by the small arrows. The large arrow points to a pair of red blood cells that are negative for the antigen (*D*). Bar, 0.01 mm.

triangles) there is absence of label. Smooth muscle cells in the media of the aorta (Fig. 3, *A* and *B*) and the arteriole (*C* and *D*) also stain intensely for LB69. Two red blood cells in the lumen of the vessel in Fig. 3 *C* (note *large arrow*) do

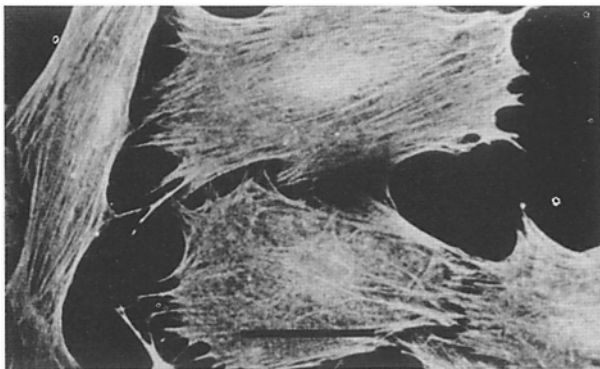


Figure 4. Immunofluorescence localization of LB69 in bovine vascular smooth muscle cells. Bovine vascular smooth muscle cells were cultured on tissue culture plastic dishes, fixed in PLP, permeabilized in 0.2% Triton X-100, then stained for LB69 using the LR2 antibodies. Bar, 0.01 mm.

not stain with the antibody, providing an internal negative control. The immunostaining of the smooth muscle cells was not due to sequestering of LB69, shed by endothelial cells, by the smooth muscle cells because cultured smooth muscle cells obtained from bovine aortic vessel walls stained for LB69 even after multiple passages in culture (Fig. 4).

Rat epididymal fat pads were isolated and either gently minced or incompletely digested with collagenase to dissociate single cells in order to demonstrate LB69 in microvascular endothelial cells. The distribution of the label was associated with vessels throughout the section. When minced fat pads were incompletely digested with collagenase to release individual cells, then stained with the antibody, there was a lack of staining in the associated connective tissues and intense staining of individual endothelial cells. The cortical staining on the majority of the cells is consistent with the notion that this molecule is found on the cell surface (data not shown).

Immunofluorescence Localization of LB69 in BAEC during *In Vitro* Sheet Migration

Large vessel endothelial cells were stained with the LR2 antibodies to investigate the distribution of LB69 during migra-

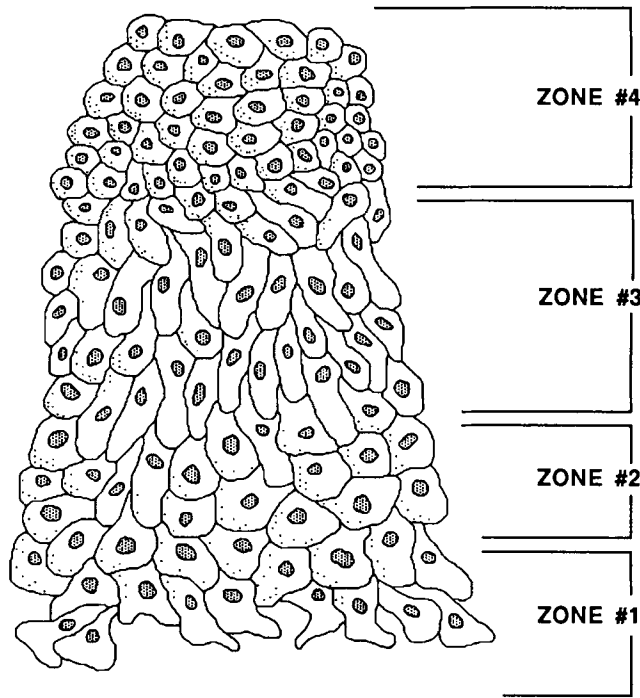


Figure 5. Endothelial cell migration. Schematic diagram of migrating endothelial cells showing the typical morphology of the cells within the various zones. *Zone 1*, the migrating front, where the cells are well spread, polar, and actively motile, displaying the typical morphology of migrating cells, *zone 2*, 5-10 cells behind the migrating front, the cells are typically flattened and well spread; *zone 3*, the cells take on an elongated appearance, aligned in the direction of migration; and *zone 4*, the center of the dish where the cells are stationary and appear as confluent cultures of endothelial cells displaying the typical cobblestone morphology of these cells (50).

tion in vitro using teflon fences previously described by Pratt et al. (34). Based on morphological criteria, we have divided the migrating sheet into various zones of migration, which is depicted in a schematic diagram (Fig. 5). BAEC were allowed to migrate onto substrate-coated dishes and at the end of the migration period the cell sheets were washed, fixed, then stained after permeabilization with 0.2% Triton X-100 in PBS or stained nonpermeabilized. In the nonpermeabilized cell cultures LB69 distribution is shown in Fig. 6, *D-F* with the phase contrast micrographs of the identical fields shown in *A-C*. Cells in zone 4 exhibit an LB69 localization pattern characterized by a prominent apical distribution noted in the majority of the cells (Fig. 6 *D*). The patches of immunoreactivity do not appear to be localized to the same areas on each cell and do not always correspond to the perinuclear region. In areas closer to the migrating front, zones 3 and 2, fewer cells demonstrate the large patch of staining. Cells in zone 2 (Fig. 6 *E*) are frequently negative and there is a proportion of the cells that have vesicular-like structures (see *arrows*) and many small irregular patches of LB69 occasionally arrayed in linear arrays across the cell body (not shown). At the migrating front (zone 1) the cells are generally devoid of any apical labeling except at ruffling borders as seen in Fig. 6 *F* where the arrows delineate the borders of a representative cell. As a control to ensure that the cells were nonpermeable, polyclonal antibodies specific for vin-

culin (Miles Laboratories, Inc.) were used and did not show any reactivity unless the BAEC were first permeabilized with 0.2% Triton-X-100 (data not shown). To insure that the fixation regiment was not compromising antigenicity, staining for angiotensin converting enzyme, which is associated with the external face of the apical plasma membrane of the endothelial cells, was performed and exhibited a characteristic pattern of fluorescence (data not shown).

In contrast, LB69 distribution in permeabilized BAEC is demonstrated in Fig. 6, *G-I*. When BAEC in zone 4 are permeabilized and then stained with the LR2 antibodies, a diffuse but granular pattern with occasional linear densities throughout the cell and at the cell edges was noted (Fig. 6 *G*). This cell edge staining is possibly due to membrane overlap and subsequent intensification of the fluorescence signal. Examination of the cells in zone 2 (Fig. 6 *H*) illustrated the redistribution of LB69 from a diffuse pattern to more continuously staining linear structures (see *arrowheads*). This redistribution is also seen at the migrating edge in zone 1 (Fig. 6 *I*). In all zones examined there is also a vesicular component to the staining pattern that appears perinuclear, possibly representing synthesis in the Golgi network.

Linear Staining of LB69 in Migrating BAEC Co-Distributes with Actin Microfilaments

Double-label studies were performed using fluorescein-labeled phalloidin to detect actin microfilaments, and rhodamine-labeled tertiary antibodies to detect LB69, in order to determine if the linear staining of LB69 co-distributes with actin microfilaments. In zone 4 the actin microfilament distribution co-distributes with LB69 (Fig. 7, *A* and *B*, respectively). Actin is seen in a dense peripheral band localization with occasional stress fibers extending the length of the cells, while LB69 appears to have a more granular pattern along the filament bundles. However, in zone 2 (Fig. 7, *C* and *D*) and zone 1 (*E* and *F*) the pattern of staining for LB69 and actin microfilaments overlaps extensively with more continuous labeling patterns along filament bundles (Fig. 7, see *arrowheads* in *C-F*).

Distribution of LB69 in BAEC Grown on Different Matrix Components

To determine whether the substrate on which the cells are grown can influence the distribution of LB69 in BAEC, cells were allowed to migrate on either types IV or I/III collagen, Lm, or Fn as described in Materials and Methods. At day 5 the cells were fixed and permeabilized then stained with the LR2 antibodies. The distribution of LB69 in zone 4 was similar if not identical on all matrix components examined. Fig. 8 shows such cells on Lm (*A*) and IV (*B*) for comparison. In zone 1, the cells on all matrices looked identical and only Lm (Fig. 8 *C*) and IV (*D*) are shown for comparison. This finding was noted with nonpermeabilized cells as well as permeabilized cells (data not shown). The changes in the distribution of LB69 appear to be due to the state of migration of the cells and not the composition of the underlying matrix.

We must consider, however, that the cells had been in culture for 5 d during which time they had been synthesizing and depositing their own matrix which may have its own

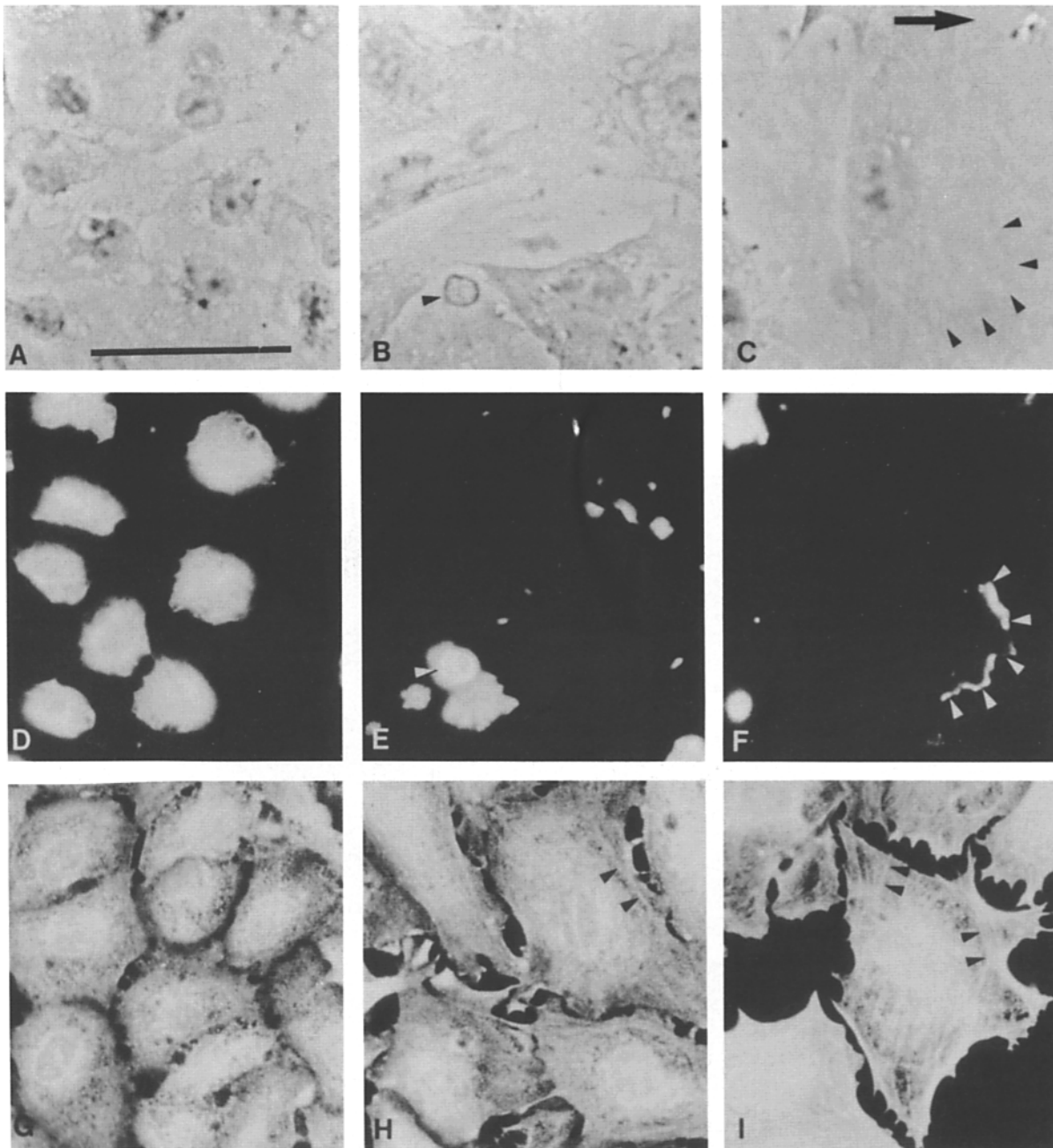


Figure 6. Immunostaining of BAEC with the LR2 monoclonal antibodies in an in vitro migration assay, migrating on a 1:1 mixture of collagen types I and III. (A–F) The cells have been fixed on ice in PLP for 90 min without permeabilization. (G–I) The cells have been fixed on ice in PLP for 90 min, then permeabilized in 0.2% Triton X-100 in PBS. The direction of migration is shown to be from left to right by the large arrow in C. A, D, and G are cells in zone 4; B, E, and H are cells in zone 2; and C, F, and I are cells in zone 1 at the migrating front. A, B, and C are phase contrast micrographs of BAEC migrating on I/III; D, E, and F are the same cells viewed with immunofluorescence labeling for LB69 with the LR2 antibodies. Note the change in morphology of the cells from a cobblestone appearance and being closely associated in zone 4, to being elongated and more spread out in zone 2, and well spread in zone 1, showing the typical morphology of a migrating cell. The staining for LB69 tends to be clustered on the apical surface of cells in zone 4 and gradually disappears in zone 2 where reactivity is seen in small patches and in vacuole-like structures (see arrowhead) until the immunoreactivity is seen restricted to ruffling membranes outlined by the arrowheads in C and F. (G–I) Permeabilized cells were stained for LB69; double arrows indicate linear arrays of LB69. Bar, 0.01 mm.

effect on LB69 distribution. Therefore, we also examined LB69 distribution during initial cell attachment and spreading, and demonstrated that the distribution of LB69 was again similar if not identical in cells attaching on I/III, IV, Lm, or Fn (data not shown).

Colocalization of LB69 with Actin in RFC and Modulation of the Distribution over Time

RFC were plated onto 35-mm Bac-T dishes (Falcon Labware, Oxnard, CA) coated with purified matrix components and allowed to attach and spread for 6 h and 96 h (conflu-

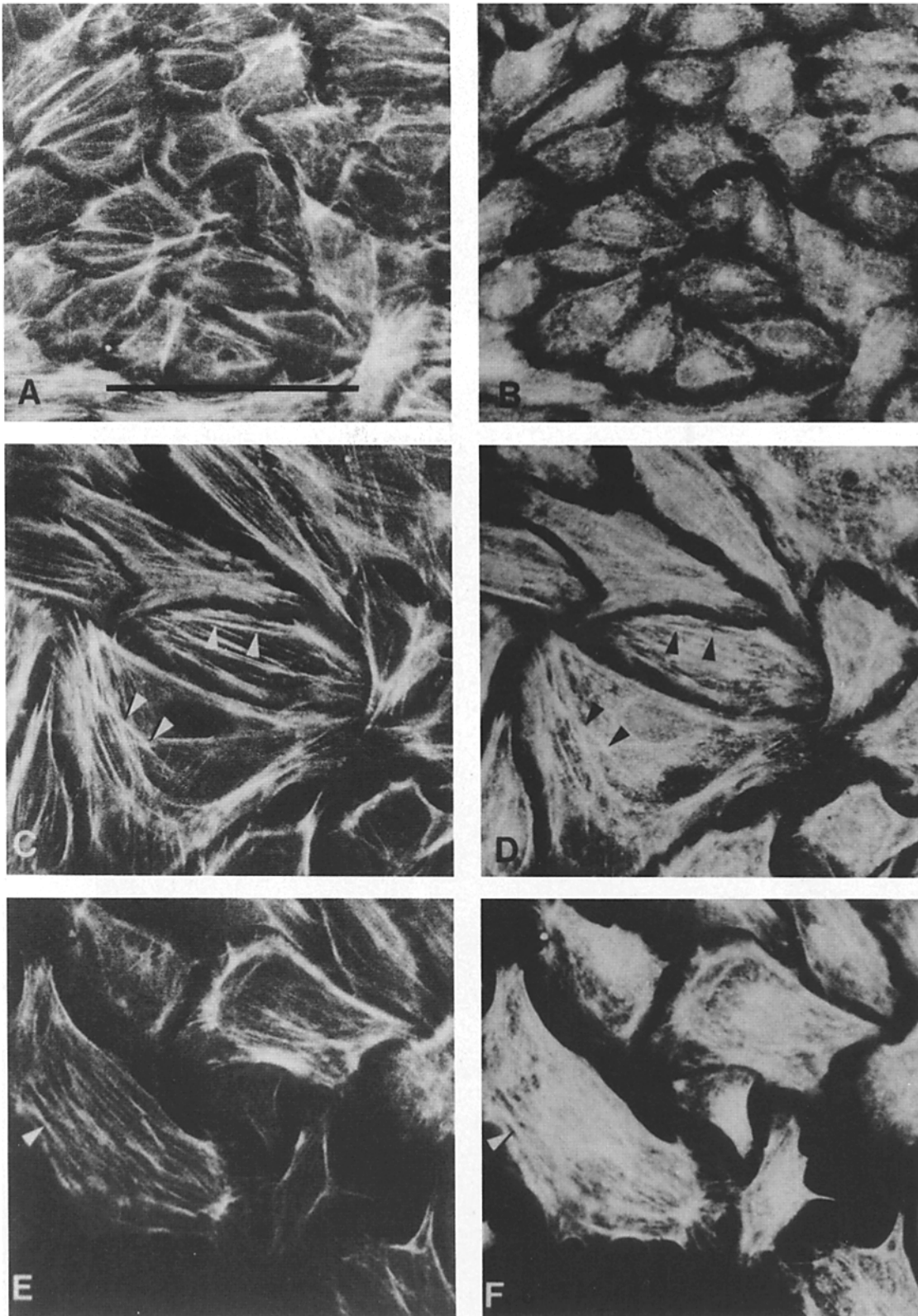


Figure 7. Immunofluorescence localization of LB69 with the LR2 monoclonal antibodies (*B*, *D*, and *F*) and actin using fluorescein-phalloidin (*A*, *C*, and *E*) in permeabilized BAEC migrating on Lm. *A* and *B* are representative cells in zone 4; *C* and *D* are representative cells in zone 2; *E* and *F* are representative cells in zone 1. Arrows indicate areas of co-localization between actin microfilaments and linear arrays of LB69. Bar, 0.01 mm.

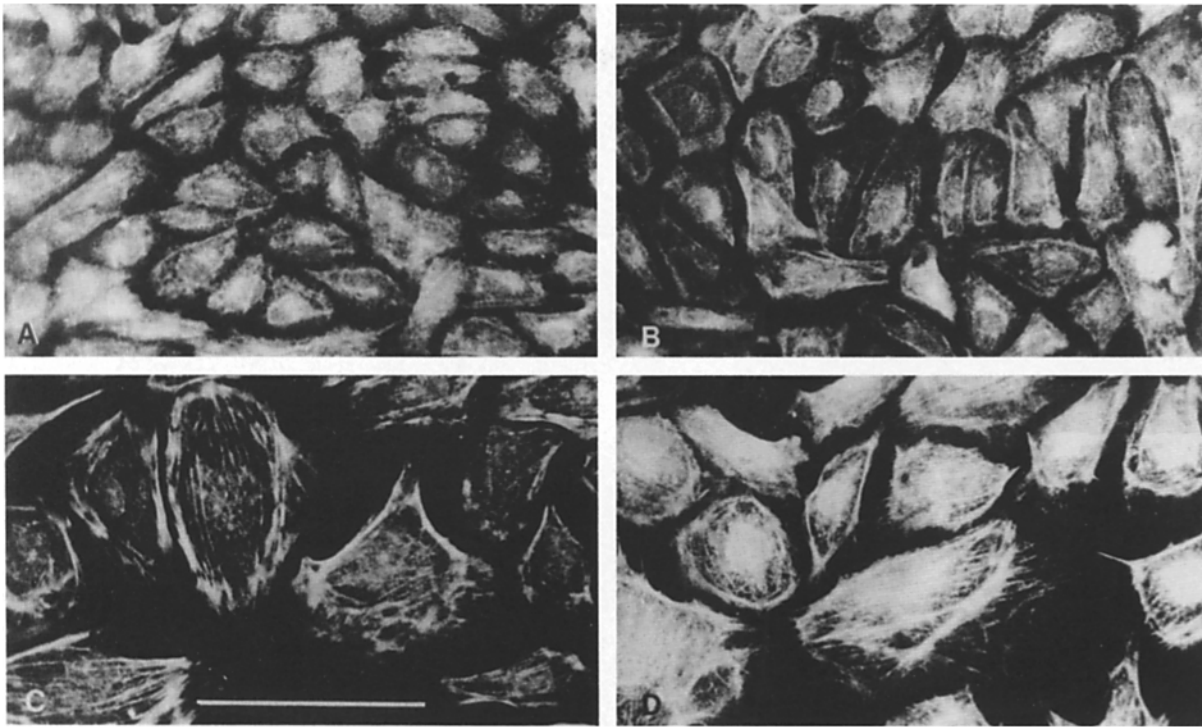


Figure 8. Immunolocalization of LB69 using the monoclonal antibodies LR2 in permeabilized BAEC migrating on Lm or IV (*A* and *B*, *C* and *D*, respectively). BAEC were fixed and permeabilized as described in Materials and Methods and stained for LB69 with the LR2 monoclonal antibodies. *A* and *C* represent cells in zone 4 of migration and *B* and *D* represent cells in zone 1. Examination of the panels demonstrates that LB69 localization in the cells is similar, if not identical, on the different matrices. Bar, 0.01 mm.

ency), then fixed, permeabilized, and stained with the LR2 antibodies and fluorescein phalloidin in double-label experiments. Fig. 9 compares LB69 distribution in RFC grown on Lm or I/III at 6 h. The antigen is distributed in linear arrays in a continuous fashion with a minor diffuse perinuclear component to the staining (Fig. 9, *B* and *D*). Double-label experiments with labeled phalloidin demonstrated a co-distribution of the linear arrays of LB69 with actin microfilaments (Fig. 9, *A* and *C*). When examined at 96 h (confluency) LB69 was still localized to linear arrays co-distributing with actin microfilaments; however, the distribution was no longer continuous. At this time point clusters of immunoreactivity along the length of the filaments were observed, with a minor component seen as clusters in between the filaments (Fig. 10). This punctate appearance contrasts the continuous distribution seen during the initial stages of spreading. Matrix specific distribution differences in RFC under these growth conditions were not observed.

Colocalization of LB69 with Lm in RFC and BAEC

LB69 could be demonstrated to co-localize with Lm on the light level in RFC cultured for 24 h on all matrix components we have examined. Fig. 11 shows RFC grown on I/III (*A* and *B*) and Fn (*C* and *D*). When stained for Lm (Fig. 11, *A* and *C*) a prominent perinuclear staining representing synthesis in the Golgi apparatus, linear arrays of staining and amorphous patches representing extracellular deposition were observed. The linear structures co-distributed with actin in double-label studies (data not shown). Lm staining in RFC grown on Lm could not be evaluated because our polyclonal

antibody is not species specific. Co-distribution of Lm with LB69 in migrating BAEC was not as readily demonstrable. However, this phenomenon could be observed in cells during migration in zone 2 where the cells are well spread and secreting small quantities of matrix (data not shown).

Discussion

Endothelial cells synthesize and interact with a complex basement membrane in which Lm is an integral component. Lm has been shown to modulate certain endothelial cell functions, specifically, Lm elicits low rates of attachment and spreading, an intermediate level of migration compared with other matrix components, and a high proliferative rate (12, 26, 28). The mechanism(s) by which endothelial cells interact with and interpret the information inherent in the structure and molecular organization of Lm in the matrix are as yet unknown. Therefore, studies were undertaken to identify cell surface matrix binding proteins for Lm that may play a role in BAEC-matrix interactions.

Lm-Seph. affinity chromatography was used to identify receptors/binding proteins for Lm in solubilized BAEC membrane preparations. We demonstrated the isolation of a 69-kD major Lm-binding protein which we refer to as LB69 for Lm binding 69-kD protein, using affinity chromatography techniques. Elution of specific Lm-binding proteins from the Lm-Seph columns could not be accomplished with low or high ionic strength salt buffers and was only accomplished with urea-containing buffers. The molecular mass corresponds to the molecular mass range of other Lm-binding proteins found in other cell systems (22, 31, 36). However,

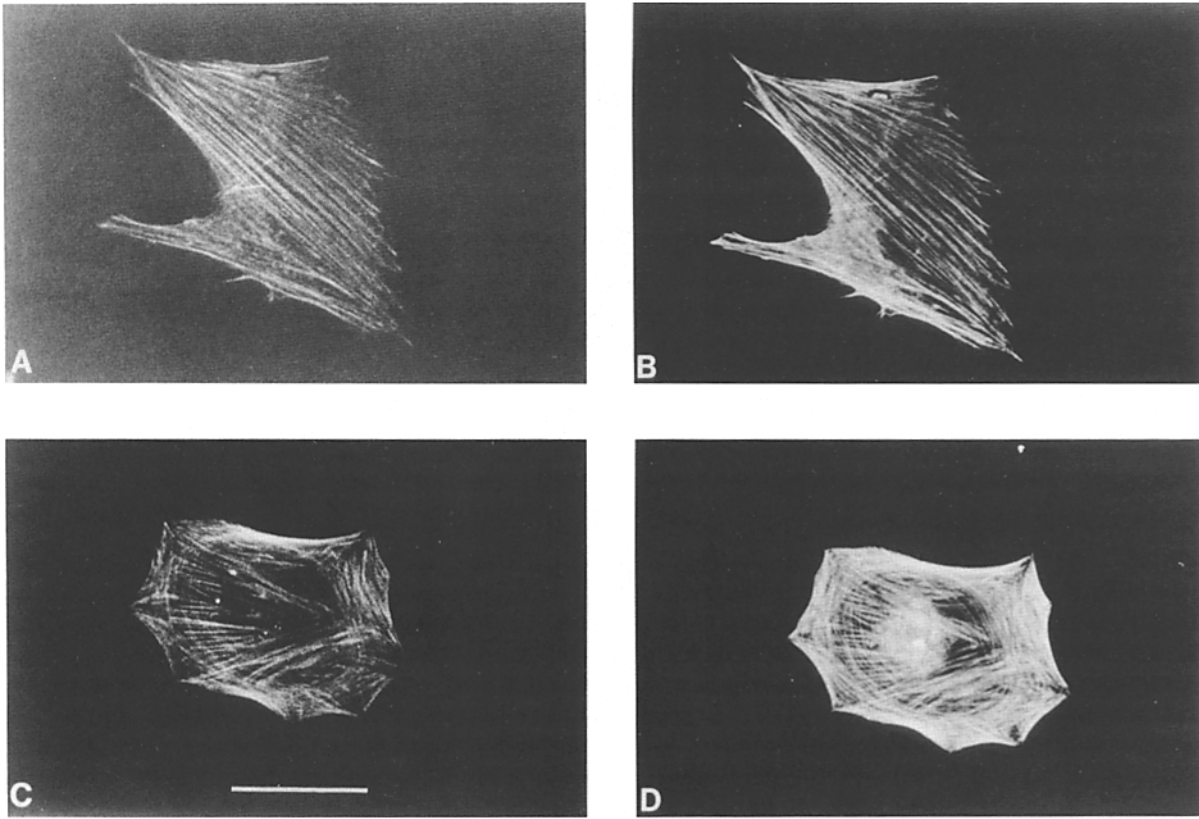


Figure 9. Double-label immunofluorescence localization of LB69 (*B* and *D*) and actin microfilaments (*A* and *C*) in RFC that had been attaching on Lm or I/III for 6 h using the LR2 monoclonal antibodies and fluorescein-phalloidin. The cells were fixed in 3.5% PFA in PBS followed by permeabilization in 0.2% Triton X-100 in PBS. *A* and *B* are identical RFC stained for the indicated antigens after attaching to a I/III substrate. *C* and *D* are the identical RFC stained for the indicated antigens after attaching to a Lm substrate. Bar, 0.01 mm.

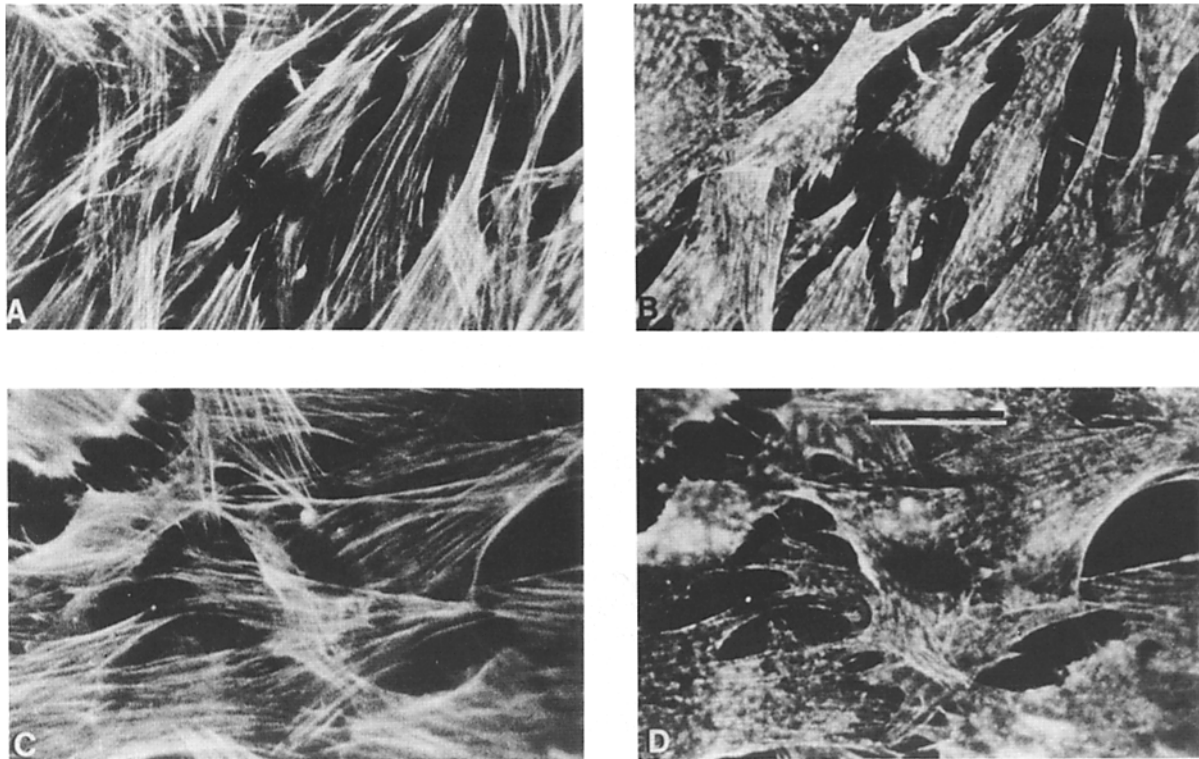


Figure 10. Double-label immunofluorescence localization of LB69 (*B* and *D*) and actin microfilaments (*A* and *C*) using the LR2 monoclonal antibodies and fluorescein-phalloidin, respectively, on confluent RFC. RFC were placed in culture for 96 h then fixed in 3.5% PFA in PBS then permeabilized with 0.2% Triton X-100 in PBS. Cells grown on a substratum composed of I/III (*A* and *B*) and cells grown on a substratum composed of Lm (*C* and *D*). Bar, 0.01 mm.

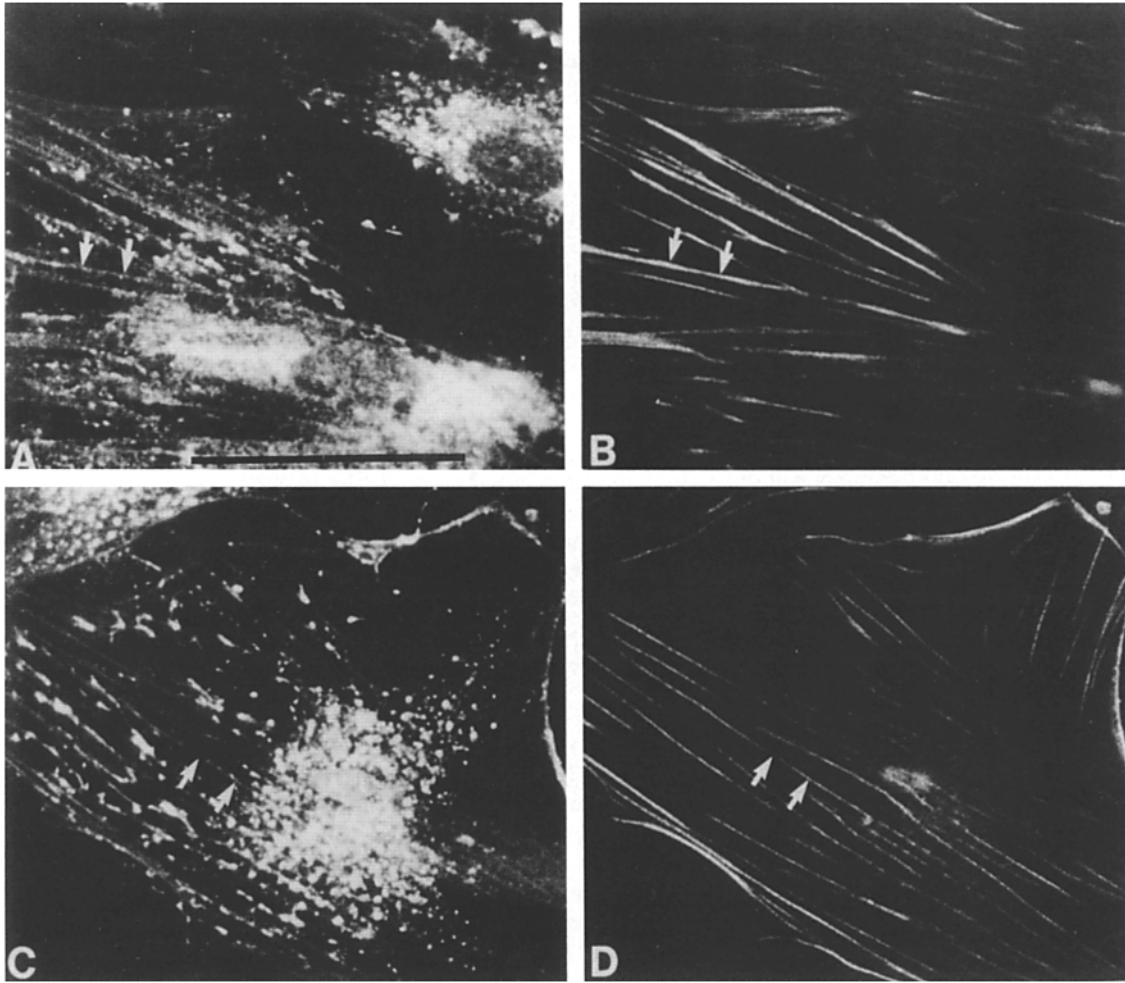


Figure 11. Double-label immunofluorescence localization of LB69 and Lm in RFC 24 h after plating. RFC were plated onto Bac-T dishes coated with on 1/III (A and B) or Fn (C and D), and allowed to attach and spread for 24 h before being fixed, permeabilized, and stained for both Lm (A and C) and LB69 (B and D). Double arrows indicate selected linear arrays of LB69 and Lm that demonstrate a prominent co-distribution. Bar, 0.01 mm.

we also note the presence of other molecular mass species coeluting with LB69. This is in contrast to what has been found in other cell systems where only one species in the range of 65–72 kD have been isolated (22, 31, 36). These differences may be due to: (a) our binding conditions use physiological salt concentrations, allowing for both high and low affinity binding proteins; (b) endothelial cells interact in vivo with a variety of ECM components under many conditions, therefore, the cells may use a variety of Lm-binding proteins to adapt to changing environments and be able to modulate their behavior accordingly; and (c) endothelial cells may have binding proteins that are specific for different regions/domains of Lm, which would transmit unique biological instructions.

The higher molecular mass gel bands correspond in molecular mass to other Lm-binding proteins previously reported in other cell systems. Specifically, the cluster of bands migrating at 92–125 kD could be related to the integrin family (cell surface adhesion molecule Ag) (17) which has been demonstrated by Horwitz et al. to bind to Lm as well as Fn in an in vitro binding assay (16). Although the mammalian (human) equivalent is believed to lack Lm binding (35), the

BAEC equivalent may be unique in this regard. The 92-kD species has a similar mobility to the GPIIIa component of the platelet GPIIb/IIIa complex (11). A GPIIIa-like molecule has been demonstrated on endothelial cells and has been shown to have the capacity to bind Fn (4, 41). GPIIIa is related to the β species of the integrin family of proteins and the VLA antigens (17, 19, 43) and antisera to the β_1 chain of VLA antigen can inhibit cell attachment to both Fn and Lm (42). Although the exact nature and relationship of these bands awaits further characterization, we have noted that BAEC express the β_1 , β_3 , and α_{Fn} chains of integrins (Basson, C. T., and J. A. Madri, manuscript in preparation).

Polyclonal antibodies directed against the 69-kD human Lm-binding protein from breast carcinoma tissue were used to demonstrate the presence of this molecule in endothelial cell lysates. Immunoblotting experiments with the polyclonal antibodies demonstrated an immunoreactive species at 69 kD in BAEC lysates demonstrating an antigenic relationship between human and bovine 69-kD protein. Attempts to demonstrate LB69 in immunoblots of BAEC lysates or solubilized membranes using the monoclonal LR2 antibodies were unsuccessful, even though the antibodies reacted

with a 69-kD band in immunoblots of human platelets (data not shown). The species differences may be such that the epitope recognized by the LR2 antibodies on the bovine protein is sensitive to nitrocellulose denaturation. However, both the polyclonal and monoclonal antibodies, as well as the synthetic peptide YIGSR, partially inhibited BAEC attachment to laminin-coated dishes. This partial inhibition of attachment elicited by the antibodies and the amino acid sequence YIGSR of the β_1 chain of laminin, thought to mediate cell attachment (13), is consistent with the participation of the 69-kD laminin-binding protein in BAEC attachment. The more dramatic inhibition of BAEC attachment on laminin-coated dishes in the presence of RGDS-containing peptides (70 vs. 20%) can be explained by laminin binding to integrins on the BAEC surface. This explanation is supported by the finding of an RGD sequence in the laminin molecule (Sasaki, M., Y. Yamada, and G. R. Martin, personal communication) and our immunoblotting studies (Basson, C. T., and J. A. Madri, manuscript in preparation). Thus, BAEC attachment to laminin appears to be complex, involving the 69-kD laminin-binding protein as well as the integrin family of proteins.

Polyclonal and LR2 monoclonal antibodies were used to probe the distribution of LB69 in aortic and microvascular endothelial cells. In all studies the polyclonal and monoclonal probes yielded identical results and the results of the monoclonal antibody labeling are illustrated in this report. The antibodies were first used to document the presence of the antigen in both aorta and epididymal fat pads. In both cases the antibody-stained endothelial cells, vascular smooth muscle cells but not the basal lamina or associated connective tissues. The staining pattern appeared to be cortical in individual endothelial cells which is consistent with a plasma membrane association. LB69 immunolocalization in aortic endothelial cells in Fig. 3 B gives the impression of apical and basolateral staining. However, the extremely flat morphology of endothelial cells makes determining topological localizations at the light level difficult.

When the distribution of LB69 was investigated in nonpermeabilized cultured BAEC during migration there appeared to be apical staining in large circular patches in zone 4. However, when the cells at or near the migrating front were studied, 69 kD staining was detected at the migrating front (zone 1) restricted to ruffling membranes. Cells in zones 2 and 3 displayed a progressive loss of the circular patches of staining which was now seen as smaller patches on the cell surface, occasionally arranged in orderly rows. Modulation of a matrix binding protein during migration is also seen with a 70-kD hyaluronic acid binding protein (HABP) isolated from fibroblasts (46, 47). HABP localizes to the dorsal surface of stationary nonmotile cells in a diffuse pattern. However, HABP was restricted to ruffling borders, the trailing edge, and a perinuclear cap in actively migrating cells (48).

The immunolocalization of LB69 to the apical surface of BAEC is surprising, since one would expect the molecule to localize basolaterally or basally. Data collected with epithelial cells grown on the BM aspect of the amnion demonstrate that LB69 is found on the basal aspect of the cells exclusively (Liotta, L., unpublished observations). Possible explanations for the presence of apical patches of LB69 could be due to: (a) incomplete polarization of the cells in the tissue culture conditions chosen or (b) the presence of Lm, which can be demonstrated in this culture system by immunofluores-

cence to be on the apical surface in zone 4 and between cells in zones 2 and 3, but never on cells in zone 1 (data not shown) and could direct the binding protein to the apical surface. From these studies, it cannot be determined whether LB69 resident on the apical surface was being removed, as the cells begin to migrate, by an active patching and internalization mechanism similar to that described for cross-linked concanavalin A receptors (14, 15); or whether clusters of LB69 accumulate on the surface of the cells as the cells become stationary and express Lm on their apical surfaces.

LB69 co-distribution with Lm could be demonstrated in subconfluent RFC and BAEC cultures. This phenomenon has also been observed in other tissue culture cells for heparan sulfate proteoglycan (51) and Fn (5, 6). Whether the molecules are participating in organizing, depositing, or internalizing the matrix is unknown. It is important to note that in our culture system, LB69 is never seen to be associated with extracellular Lm that is not cell associated.

Cytoskeletal associations have been reported in the literature for many of the previously described matrix binding proteins. The integrin complex has been shown to associate with talin, a cytoskeletal associated protein, using equilibrium gel filtration (16). This cytoskeletal association has also been demonstrated on the light level using immunofluorescence techniques, and a co-distribution between 140-kD complex and actin microfilaments has also been documented (6). The human Lm-binding protein LB69 from a breast carcinoma cell line has been shown to cosediment with actin microfilaments in an *in vitro* assay (3) and this same molecule is found associated with the detergent insoluble cytoskeleton after cross-linking cell surface bound Lm with antibodies (7). Another example includes an integral membrane heparan sulfate proteoglycan that has been demonstrated to co-distribute with actin microfilaments in breast epithelial cells by indirect immunofluorescence (37, 51). Therefore, it was not surprising when a co-distribution between LB69 and actin microfilaments was demonstrated in Triton X-100-permeabilized endothelial cells under certain culture conditions. BAEC stained at confluence showed actin in dense peripheral bands and in stress fibers and the LB69 in a diffuse pattern with co-distribution at the dense peripheral bands. During migration one sees a redistribution of the LB69 staining into linear structures that co-distribute with actin microfilaments. Permeabilized cultured RFCs also displayed a light level co-distribution with microfilament bundles and can modulate that distribution over time. The ability of BAEC and RFC to modulate the association of LB69 with microfilaments and the distribution of LB69 along microfilaments suggests that if a transmembrane connection exists between LB69 and microfilaments the association is dynamic and other cytoskeleton elements and regulatory elements, as yet to be defined, may have potential interactions with the receptor.

We have described a 69-kD Lm-binding protein in both large and microvascular endothelial cells. Immunochemical techniques localize this protein to the cell surface and double-label studies demonstrate a possible association of this molecule with actin microfilaments. The topological localization and the cytoskeletal associations can be modulated by the cell during different cellular phases. How the endothelial cell controls the intracellular movements and association of LB69 are not known. Further investigations of

this Lm-binding protein will provide us with a number of insights regarding cell-matrix interactions.

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