Spatiotemporal Segregation of Endothelial Cell Integrin and Nonintegrin Extracellular Matrix-binding Proteins during Adhesion Events

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Abstract. Bovine aortic endothelial cell (BAEC) attachments to laminin, fibronectin, and fibrinogen are inhibited by soluble arginine-glycine-aspartate (RGD)–containing peptides, and YGRGDSP activity is responsive to titration of either soluble peptide or matrix protein. To assess the presence of RGD-dependent receptors, immunoprecipitation and immunoblotting studies were conducted and demonstrated integrin $\beta1$, $\beta3$, and associated α subunits as well as a $\beta1$ precursor. Immunofluorescence of BAECs plated on laminin, fibronectin, and fibrinogen reveals different matrix-binding specificities of each of these integrin subclasses. By 1 h after plating, organization of $\beta1$ integrin into fibrillar streaks is influenced by laminin and fibronectin, whereas $\beta3$ integrin punctate organization is influenced by fibrinogen and the integrin spatial distribution changes with time in culture. In contrast, the nonintegrin laminin-binding protein LB69 only organizes after cell–substrate contact is well established several hours after plating. Migration of BAECs is also mediated by both integrin and nonintegrin matrix-

binding proteins. Specifically, BAEC migration on laminin is remarkably sensitive to RGD peptide inhibition, and, in its presence, $\beta1$ integrin organization dissipates and reorganizes into perinuclear vesicles. However, RGD peptides do not alter LB69 linear organization during migration. Similarly, agents that block LB69—e.g., antibodies to LB69 as well as YIGSR-NH$_2$ peptide—do not inhibit attachment of nonmotile BAECs to laminin. However, both anti-LB69 and YIGSR-NH$_2$ inhibit late adhesive events such as spreading. Accordingly, we propose that integrin and nonintegrin extracellular matrix–binding protein organizations in BAECs are both temporally and spatially segregated during attachment processes. High affinity nonintegrin interaction with matrix may create necessary stable contacts for longterm attachment, while lower affinity integrins may be important for initial cell adhesion as well as for transient contacts of motile BAECs.

E NDOTHELIAL cells are generally nonproliferative stationary polar cells that create a highly metabolic nonthrombogenic luminal surface within blood vessels. In intimate contact with the basal aspect of the endothelium is a complex basement membrane comprised of a variety of glycoproteins and proteoglycans (Madri et al., 1980a). In response to an injury, such as balloon catheter denudation, endothelial cells migrate over and proliferate on this matrix to reconstitute a nonthrombogenic vascular lining (Haudenschild and Schwartz, 1979). The matrix, itself, is a dynamic surface whose composition changes in response to synthetic and degradatory properties of endothelial, smooth muscle, and inflammatory cells (Pratt et al., 1985).

In vitro, extracellular matrix components such as fibronectin (Fn),[1] laminin (Ln), interstitial collagens, and basement

membrane collagens can all modulate the shape and behavior of bovine aortic endothelial cells (BAECs) (Madri et al., 1988). BAEC–substratum interaction may differ from many other cell systems since workers have long recognized that chelators such as EDTA are insufficient to detach BAECs from tissue culture vessels, and even routine tissue culture requires a combination of trypsin and EDTA (Pratt et al., 1984). Nevertheless, the cellular binding proteins for extracellular matrix proteins that investigators have documented in other systems may also be potential mediators of BAEC interaction with matrix elements (Rao et al., 1983; Van Mourik et al., 1985; Ruoslahti and Pierschbacher, 1987). In earlier studies (Yannariello-Brown et al., 1988), this laboratory has demonstrated the presence of a previously described

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1. *Abbreviations used in this paper:* BAEC, bovine aortic endothelial cell; Fb, fibrinogen; Fn, fibronectin; HIFCS, heat-inactivated FCS; HUVEC, human umbilical vein endothelial cell; Ln, laminin; Sulfo-MBS, maleimidobenzoyl-sulfosuccinimido ester.

69-kD Ln-binding protein (Rao et al., 1983; Malinoff and Wicha, 1983; Wewer et al., 1987; Yow et al., 1988) on the BAEC surface. This protein, referred to as LB69, exhibits an organization modulated by extracellular Ln, can be radioactively labeled at the cell surface, and binds to Ln immobilized on Sepharose (Yannariello-Brown et al., 1988). Some investigators have suggested that LB69 may bind Ln via a B1 chain tyrosine-isoleucine-glycine-serine-arginine (YIGSR) sequence (Graf et al., 1987a,b). However, the observation that LB69 is not the only species purified by such Ln affinity chromatography (Yannariello-Brown et al., 1988) suggested the existence of other Ln-binding proteins. Furthermore, the relative specificity of LB69 for Ln implied the existence of different binding proteins for other matrix components such as Fn and fibrinogen (Fb).

The "integrin" class of proteins are good candidates for such binding proteins (Hynes, 1987). Integrins have been shown to bind largely in a divalent cation–dependent manner to extracellular matrix proteins such as Fn, Fb, and Ln via an arginine-glycine-aspartate (RGD) ligand sequence (Ruoslahti and Pierschbacher, 1986). This sequence appears in a presumed β turn of Fn in the sequence GRGDSP (Pierschbacher and Ruoslahti, 1984) and in the Ln A chain as well (Sasaki et al., 1988). Fb also contains such an RGD sequence (Plow et al., 1985). Previously described integrin receptors (Buck et al., 1986; Tamkun et al., 1986; Argraves et al., 1987; Fitzgerald et al., 1987a,b; Hynes, 1987) are comprised of two disulfide-linked subunits, an α and smaller β, and the molecular weights of several bands purified from BAECs by Ln affinity chromatography in addition to LB69 (Yannariello-Brown et al., 1988) are not inconsistent with those of integrin subunits. Moreover, others have documented that two classes of integrins, referred to as β1 and β3 for their class of β subunits (Hynes, 1987), are present in human umbilical vein endothelium (Plow et al., 1986; Newman et al., 1986; Charo et al., 1987; Cheresh, 1987; Fitzgerald et al., 1987b; Dejana et al., 1988a,b) and in rat liver endothelial cells (Johansson et al., 1987) and they have noted their involvement in cell attachment to extracellular matrix. Fitzgerald et al. (1985) and Hayman et al. (1985) have identified β3 integrins in bovine aortic endothelium.

In this communication, we have used RGD peptides and specific antisera to integrins to demonstrate that BAECs do express β1 and β3 class integrin proteins and that they participate in BAEC attachment to Ln, Fn, and Fb. Furthermore, despite sharing a common ligand sequence (RGD) these integrins possess certain binding specificities for their extracellular matrix ligands which can in turn modulate integrin organization. We also confirm that nonintegrin-dependent mechanisms contribute to BAEC adhesion to extracellular matrix and we show that integrin and nonintegrin BAEC proteins exhibit a spatiotemporal segregation during attachment, spreading, and migration on Ln.

Materials and Methods

Cells

BAECs were harvested and cultured from bovine calf aortae as previously described (Madri et al., 1980a; Yannariello-Brown et al., 1988). BAECs used for these studies were all between passages 8 and 14.

For certain experiments, FCS was replaced with either dialyzed heat-inactivated FCS (HIFCS) or defibronectinized HIFCS. Defibronectinized HIFCS was produced by adsorbing Fn from the HIFCS by incubation overnight at 4°C with gelatin-Sepharose and then by further passing the serum over an affinity matrix of anti-Fn antibody coupled to Sepharose Cl-4B (Pharmacia Fine Chemicals, Piscataway, NJ). Quantitative ELISA inhibition assay (Madri et al., 1988) with specific anti-Fn antibodies demonstrated that this defibronectinized HIFCS contained <0.2 ng/ml Fn.

Matrix Proteins

Plasma Fn was isolated and purified as previously described (Madri et al., 1980b; Yannariello-Brown et al., 1988). Ln was isolated from the Engelbreth-Holm-Swarm tumor grown subcutaneously in lathrytic mice as previously described by Yannariello-Brown et al. (1988). ELISA with antibodies for entactin (generous gift of Drs. H. Steiner and J. Lwebuga-Mukasa, Yale School of Medicine, New Haven, CT) demonstrated that the preparation contained only 3% contaminating entactin, and resultant coating concentrations of entactin are not sufficient to support any cell attachment. Fb was purchased from Calbiochem-Behring Corp. (La Jolla, CA). ELISA with antibodies to Fn demonstrated that this Fb preparation contained only 0.8% Fn.

Synthetic Peptides

Peptides, YGRGDSP, YGRGESP, YGRGDSPC, and YIGSR-NH$_2$, were synthesized on a synthesizer (403A; Applied Biosystems, Inc., Foster City, CA) using standard t-butoxycarbonyl chemistry and were deprotected and released from their solid-phase support by using hydrogen fluoride methods with appropriate scavengers. Purification of peptides was done by reverse-phase HPLC on a Dynamax Macro C18 preparative column (Rainin Instrument Co. Inc., Woburn, MA) with a trifluoroacetic acid and acetonitrile gradient, and residual trifluoroacetate was removed from peptides by exchange for chloride salts. Composition of all peptides was confirmed by amino acid analysis. In addition, for the YIGSR-NH$_2$ peptide, methylbenzhydrylamine resin (Applied Biosystems, Inc.) was used to synthesize the amide form of the peptide, and the peptide composition and sequence and the presence of COOH-terminal amide were confirmed with mass spectrophotometry by Drs. L. Haddad, P. Lyons, and S. Hunt (3M Corp., Minneapolis, MN). Peptide concentrations were precisely determined by measuring the optical density at 274 nm of peptide solutions and by using a specific extinction coefficient for tyrosine at this wavelength.

The COOH terminus of YGRGDSPC peptide was coupled to keyhole limpet hemocyanin (Calbiochem-Behring Corp.) via a maleimidobenzoyl-sulfosuccinimido ester linker (sulfo-MBS; Pierce Chemical Co., Rockford, IL) by the instructions of the manufacturer. Assay (Anderson and Wetlaufer, 1975) with dithio-bis-(2-nitrobenzoic acid) (Pierce Chemical Co.) demonstrated four to six active thiol groups on the keyhole limpet hemocyanin linker complex before the addition of peptide. Similar procedures were used to couple YGRGDSPC via sulfo-MBS to Sepharose Cl-4B (Pharmacia Fine Chemicals) with a hexane diamine spacer arm (Hashida et al., 1984).

The COOH terminus of YGRGDSP peptide was coupled to BSA (Sigma Chemical Co., St. Louis, MO) via an 1-ethyl-3-(3-dimethylpropyl)-carbodiimide (Sigma Chemical Co.)–mediated condensation reaction using the methods of Tamura et al. (1983). Affinity-purified antibodies to YGRGDSPC, generated as described below, were used in a quantitative ELISA inhibition assay to quantitate the amount of synthetic peptide in the YGRGDSP-BSA complex, and YGRGDSP was shown to be in a 90:1 molar ratio to BSA.

Antibodies

Rabbits were immunized at 30–35 sites with a YGRGDSPC-keyhole limpet hemocyanin complex (described above) and boosted 4 wk later. Bleeding was performed 7 wk after primary injection. Antibodies specific for YGRGDSPC synthetic peptide were affinity purified with blocked sulfo-MBS-Sepharose Cl-4B and YGRGDSPC-sulfo-MBS-Sepharose Cl-4B resins (described above). Eluted antibody was shown in an ELISA assay to be specific for the YGRGDSP and YGRGDSPC peptides with a greatly decreased (more than one order of magnitude) affinity for the YGRGESP peptide. Affinity-purified rabbit antibodies to human plasma Fn and Engelbreth-Holm-Swarm Ln were prepared and purified as previously described (Madri et al., 1980b). Anti-Fb rabbit IgG was purchased from Cappel Laboratories (Cochranville, PA).

Polyclonal rabbit antisera to chicken CSAT antigen band 3 (anti-GP3; Damsky et al., 1985), to rat integrin (anti-R140; Buck and Horwitz, 1987),

and to a β1 integrin cytoplasmic domain 10-amino acid synthetic peptide WDTGENPIYK (anti-β1/10P; Buck, C., personal communication) were all generous gifts of Dr. C. Buck (Wistar Institute, Philadelphia, PA). Polyclonal rabbit antibody (anti-GPIIIa) and monoclonal mouse antibody (22C4; Plow et al., 1986) to human platelet GPIIIa were graciously donated by Dr. Mark Ginsberg (Research Institute of Scripps Clinic, La Jolla, CA). Polyclonal rabbit antisera to chicken CSAT Fn receptor (kindly donated by Dr. K. Yamada, National Cancer Institute, Bethesda, MD), to the hamster Fn receptor (anti-GP140; donated by Dr. C. Damsky, University of California at San Francisco, San Francisco, CA; Knudsen et al., 1981), and to the VLA β protein (Takada et al., 1987a; donated by Dr. M. Hemler, Dana Farber Cancer Institute, Boston, MA). Polyclonal antiserum was raised against human adult large vessel endothelium IIb/IIIa-like protein (R838) as previously described (Albelda et al., 1989). Mouse monoclonal antibody to human p150,95 (Leu-M5) was obtained from Becton Dickinson Monoclonal Center, Inc. (Mountain View, CA). Dr. T. Springer (Harvard Medical School, Boston, MA) supplied mouse monoclonal antibody ascites TS1/22, anti-LFA-1 α subunit (TS1/22.1.1.13.3; American Type Culture Collection, Rockville, MD). Polyclonal rabbit antiserum to LB69 Ln-binding protein (anti-LB69—previously shown to recognize BAEC LB69 in vivo and in vitro (Yannariello-Brown et al., 1988)—and to an LB69 exodomain peptide (P20A, also known as 3801) were prepared and shown to exhibit similar antigenic specificities as already described (Wewer et al., 1987; Rao et al., 1989). These antisera were used in the experiments described as resources permitted. Previous work demonstrated that antibody reactivity to the 37-kD protein represents activity against an internal (cytoplasmic) antigenic site and therefore does not contribute to functional properties in live cell assays (Wewer et al., 1987; Rao et al., 1989; Castronovo, V., A. P. Claysmith, H. C. Krutzsch, and M. E. Sobel, manuscript submitted for publication).

Preparation of Matrix Protein Substrates for Cell Culture

Bacteriologic plastic 60-mm-diameter dishes (Falcon Labware, Oxnard, CA) or 24-well cluster dishes (Costar, Cambridge, MA) were saturated with matrix proteins as previously described with minor modifications (Madri et al., 1988; Form et al., 1986), and nonspecific protein binding sites were pacified with 1% heat-inactivated BSA. At protein-coating concentrations of 25 μg/ml (Ln and Fb) or 12.5 μg/ml (Fn), the cluster dish bacteriologic plastic was shown by quantitative ELISA inhibition assay to be saturated with 49 ng/cm² Fn, 78 ng/cm² Ln, 66 ng/cm² Fb. Nonsaturating coatings were produced by coating with matrix protein solutions with decreased protein concentrations, and absolute amounts of resultant protein adsorbed to the bacteriologic plastic were determined by ELISA. Dishes could also be coated with synthetic YGRGDSP peptide by incubation with 25 μg/ml solutions of YGRGDSP-BSA conjugates overnight as described above.

Acid Phosphatase Index Cell Attachment Assays

BAEC attachment was estimated by using acid phosphatase activity as an indicator of cell number with a modification of the methods described by Connolly et al. (1986). Preliminary studies showed that acid phosphatase activity was linearly related to cell number as determined by Coulter counting. BAEC cultures were treated with trypsin/EDTA (Falcon Labware) and resuspended in ice-cold DME supplemented with 10% defibronectinized FCS. 20,000 cells were plated into matrix-coated 16-mm-diameter wells of 24-well cluster dishes in the presence of varying concentrations of synthetic peptides. To study the effects of antibodies, cells were pretreated for 1 h on ice with heat-inactivated (56°C for 30 min) antibodies at a final dilution of 1:50. This dilution represents a functional titer for the antibodies studied as previously shown in Wewer et al. (1987), Damsky et al. (1985), and Albelda, S. M., and Buck, C. A. (unpublished observations). At the same time, serial dilutions of cells were plated without any peptides to provide standard curves of plating efficiency. Dishes were incubated for 1 h at 37°C and then washed twice with PBS. Acid phosphatase activity in each well was measured by incubation at 37°C for 2 or 3 h, depending on the matrix protein coating and the experimental conditions, in the presence of 200 μl of 10 mM p-nitrophenyl phosphate (Sigma Chemical Co.), 0.1% Triton X-100, 0.1 M sodium acetate, pH 5.5. Substrate hydrolysis was stopped, and the color was developed by the addition of 20 μl of 1 N NaOH. Samples were transferred to 96-well plates, and optical absorbances at 405 nm were monitored with a Titertek multiskan (Flow Laboratories, Inc., McLean, VA). By using the standard curve of cell number serial dilutions, optical densities were converted to the percentage of cells attached to the matrix-

coated plastic in the presence of peptide relative to those attached in untreated wells.

For spreading determination, cultures were prepared as above, allowed to incubate for 6 h, fixed, and stained with hematoxylin. Spreading was assessed as the percent of attached cells with spread profiles (deviation from round) in representative fields of quadruplicate wells relative to untreated controls. Cell size was measured on ~90 cells per well by morphometric analysis with a digitizing tablet and expressed as an index ratio relative to untreated controls. (Madri et al., 1988).

Cell Migration Assay

BAEC radial sheet migration over matrix-coated 35-mm-diameter dishes (Falcon Labware) was assayed by release from contact inhibition using a Teflon fence previously described (Pratt et al., 1984; Madri et al., 1988). Migration rates were expressed as square millimeters covered per day. To assess the effects of synthetic YGRGDSP peptide on BAEC migration, YGRGDSP-BSA conjugate was added to cultures on days 1 and 4 at a final peptide concentration of 5 μM in the presence of defibronectinized serum and an additional 10-fold excess of exogenous uncoupled BSA over BSA coupled to peptide. Adducts were determined by quantitative ELISA inhibition assay with affinity-purified antibody to YGRGDSP to be composed of a 90:1 molar ratio of YGRGDSP/BSA, and addition of peptide-BSA adduct in the presence of free BSA prevented substantial cellular uptake and degradation of peptide during the course of the assay. Consistent with the in vivo observations of Gehlsen et al. (1988a), we have noted the half-life of uncomplexed small RGD peptides in subconfluent BAEC cultures to be on the order of 10-15 min (data not shown). Control cultures received only uncoupled BSA in the presence of defibronectinized serum.

Indirect Immunofluorescence Analysis

Cells were fixed for 90 min at 4°C with paraformaldehyde-lysine-periodate fixative, permeabilized with 0.2% Triton X-100, and labeled with polyclonal rabbit antisera to matrix-binding proteins as described by Yannariello-Brown et al. (1988). Bound antibodies were detected with a rhodamine-conjugated goat anti-rabbit secondary antibody (Cappel Laboratories). Samples were examined on a binocular microscope (14; Carl Zeiss, Inc., Thornwood, NY) or a confocal imaging system (MRC-500; Bio-Rad Laboratories, Richmond, CA).

BAEC Lysate Preparation

BAEC cultures were chilled on ice and washed twice with PBS supplement with a protease inhibitor mixture: 1 mM diisopropyl fluorophosphate (Aldrich Chemical Co., Milwaukee, WI), 2 mM PMSF (Sigma Chemical Co.), 0.16 TIU/ml aprotinin (Sigma Chemical Co.), 0.5 μg/ml leupeptin (Sigma Chemical Co.), and 8 mM iodoacetamide (Sigma Chemical Co.). (If lysates were to be electrophoresed under reducing conditions, iodoacetamide was omitted.) Cells were removed with a teflon scraper from the dish and suspended in PBS/protease inhibitor mixture. The cells were centrifuged (2,000 rpm for 5 min at 4°C), resuspended in 2 vol of TNC buffer (0.5% NP-40, 0.5 mM CaCl₂, 10 mM Tris-acetate, pH 8.0; Horwitz et al., 1985) supplemented with protease inhibitor mixture, and stirred for 30 min on ice. The cell mixture was spun in an Eppendorf microfuge (Brinkmann Instruments, Inc., Westbury, NY), and the supernatant was decanted and represented the BAEC lysate.

Immunoblot Analysis of BAEC Lysates

Freshly prepared lysates (200-300 μl) were electrophoresed on 6% SDS-polyacrylamide gels (Laemmli, 1970) under reducing or nonreducing conditions. Protein was then transferred to 0.45-μm pore nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH) with a Polyblot apparatus (American Bionuclear, Emeryville, CA) according to the instructions of the manufacturer. Total protein was detected by staining with Amido black. Membranes were blocked with 4% BSA in PBS containing 0.05% NaN₃ overnight at 4°C. Membranes were then incubated with rabbit polyclonal antisera or mouse monoclonal antibodies diluted in blocking solution for 2 h at room temperature with gentle agitation. Subsequently, nitrocellulose strips were washed with 0.05% Tween-20 in 50 mM Tris, 150 mM NaCl, pH 7.4, containing 0.05% NaN₃ and then reacted with an alkaline phosphatase-conjugated goat anti-rabbit Fc or anti-mouse IgG secondary antibody (Promega Biotec, Madison, WI) diluted 1:7,500 in 0.05% Tween-20 in 50 mM Tris, 150 mM NaCl, pH 7.4, containing 0.05% NaN₃ for 1 h

at room temperature. Strips were rewashed and incubated with AP buffer (100 mM NaCl, 5 mM MgCl$_2$, 10 mM Tris, pH 9.5), and bound secondary antibody was detected in incubation at room temperature with a substrate solution of nitroblue tetrazolium (330 μg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (165 μg/ml) in AP buffer. Substrate hydrolysis was stopped by washing nitrocellulose with 5 mM EDTA, 20 mM Tris, pH 8.0.
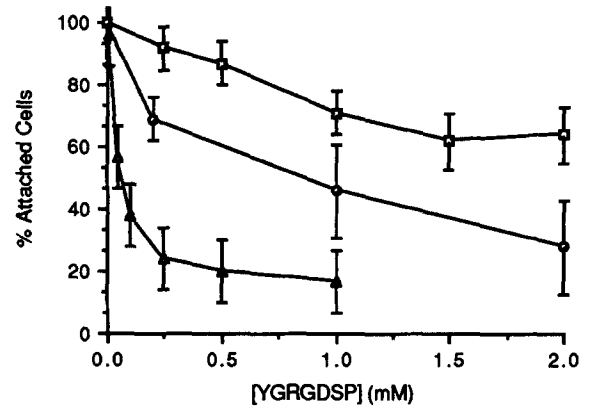
Integrin Immunoprecipitation from BAEC Lysates

BAECs were grown to subconfluence and washed with PBS. Cells to be surface labeled were treated for 10 min with 1 mCi/ml Na^{125}I (15.5 mCi/μg; Amersham Corp., Arlington Heights, IL) in PBS containing 100 U/ml lactoperoxidase (Sigma Chemical Co.) and 0.0048% hydrogen peroxide. The reaction was quenched with excess cold NaI. Cells to be metabolically labeled were incubated at 37°C for 1 h with methionine-free DME (Gibco Laboratories, Grand Island, NY) without serum. Cultures were then incubated at 37°C for 18 h with methionine-free DME supplemented with 10% dialyzed HIFCS and 83 μCi/ml L-[^{35}S]methionine (186.4 mCi/mmol; Amersham Corp.). Cells were then grown in fresh complete medium without radioactivity for varying time periods as experimental conditions required. Lysates were prepared as described above. 100 μl 4% BSA-PBS, 150 μl 2 M NaCl, and 50 μl of double-distilled water were added per milliliter of lysate. Lysates were then preabsorbed with 0.02 vol Sepharose Cl-6B by incubation with agitation for 30 min at 4°C. 50 μl of rabbit polyclonal antiserum diluted 1:50 in 4% BSA-PBS were then added to 100 μl of preabsorbed lysate and incubated with agitation for 1 h at 4°C. 20 μl of protein A-Sepharose (Pierce Chemical Co.) were added to each sample and agitated for 1 h at 4°C. The Sepharose resin with bound antigen-antibody complex was pelleted in an Eppendorf microfuge (Brinkmann Instruments Co.) and washed five times with 1 ml 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 150 mM NaCl, 50 mM Tris, pH 7.4. Sepharose was then boiled for 3 min in sample buffer (Laemmli, 1970) and then electrophoresed on 6% SDS-PAGE as above under nonreducing conditions. Gels were dried, treated with EN^{3}HANCE (New England Nuclear, Wilmington, DE), and exposed on XAR-5 film (Eastman Kodak Co., Rochester, NY).
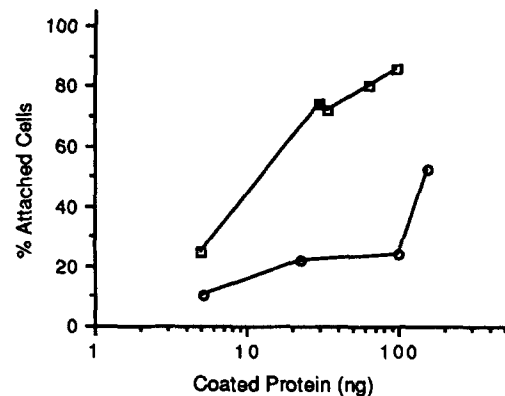
Results

Effects of Synthetic RGD Peptides on BAEC Attachment

BAEC attachment to saturating coatings of Fn, Ln, and Fb was similar at 4 h after plating, but rates of attachment during this period varied among these matrices. Specifically, at 1 h after plating, >95% of plated cells attached to Fn, and, relative to this adhesion, 93% attached to Fb but only 42% to Ln.

Moreover, BAEC attachment after 1 h to bacteriologic plastic surfaces saturated with Fn, Ln, or Fb exhibited differential sensitivities to RGD peptides. Compared with untreated controls, when BAECs were treated with 1 mM YGRGDSP during plating (n = 2-7), 77 ± 3% attached to Fn, 53 ± 2% to Ln, and only 16 ± 1% to Fb. These relative sensitivities to YGRGDSP were preserved over a broad range of peptide concentrations (Fig. 1 A), with BAEC attachment to Fb being consistently extremely susceptible to RGD inhibition. The control peptide YGRGESP had relatively no effect at a 1 mM concentration on cell attachment to any of the matrix substrates assayed. Similarly, 1 mM YGRGESP had no effect on BAEC attachment to plastic saturated with a YGRGDSP-BSA adduct, whereas 1 mM YGRGDSP completely inhibited all such attachment (data not shown). While the sensitivities of attachments to Fn and Ln to YGRGDSP are clearly different, the approximate RGD peptide doses for half-maximal inhibition of BAEC attachment to these substrates were similar (0.4 and 0.3 mM, respectively). The half-maximal dose for inhibition of attachment to Fb is approximately an order of magnitude lower at 0.04 mM (Fig. 1 A). Attachment to Fn or Ln by untreated BAECs or by those treated with 1 mM YGRGESP was largely unaffected

A [YGRGDSP] (mM)

B Coated Protein (ng)

Figure 1. Effects of YGRGDSP concentrations on BAEC attachment to Fn, Ln, and Fb. (A) Attachment assays were conducted on plastic coated with saturating amounts of Fn (squares), Ln (circles), and Fb (triangles) in the presence of a range of YGRGDSP concentrations. Note that half-maximal doses of YGRGDSP for inhibition of BAEC attachment to Fn and Ln are similar, whereas the half-maximal dose for Fb is an order of magnitude less. (B) Attachment assays were conducted on plastic coated with various concentrations of Fn or Ln in the presence of 1 mM YGRGDSP. Data is expressed as the percentage of adherent cells relative to untreated cells plated in wells coated with saturating amounts of matrix protein. The amount of matrix protein adsorbed to the plastic well was determined by quantitative ELISA inhibition assay. Inhibition of attachment was augmented at decreased coating concentrations of either Fn (squares) or Ln (circles). Data portrayed here represent means of duplicate wells in a typical assay, and standard errors do not exceed ±10%.

by alterations of substrate coating over a 3-200-ng range. When the amount of coated Fn or Ln was decreased over this same range, the effects of 1 mM YGRGDSP were greatly enhanced and fewer cells attached (Fig. 1 B). Thus, the YGRGDSP peptide was shown to compete specifically with the coated matrix protein for cellular binding sites.

Identification of BAEC Integrins and LB69 by Immunoprecipitation and Immunoblot Analysis

Lysates of BAECs surface labeled with ^{125}I were immunoprecipitated with anti-integrin antibodies and analyzed elec-
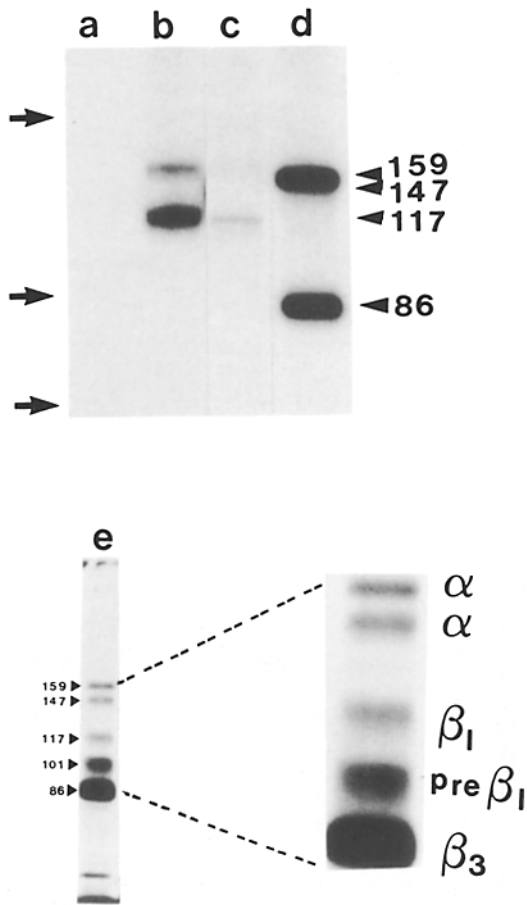
Figure 2. Immunoprecipitation of integrin molecules from BAEC lysate. BAECs were grown to subconfluence on tissue culture plastic and either surface labeled with ^{125}I (lanes *a–d*) or metabolically labeled for 18 h with [^{35}S]methionine (lane *e*). Lysates were prepared, and proteins were immunoprecipitated with nonimmune serum (lane *a*), anti–R140 (lanes *b* and *e*), anti–GP3 (lane *c*), or R838 (lane *d*). Arrows indicate molecular mass markers of 212, 97, and 67 kD in decreasing order, and arrowheads indicate the molecular masses of the major immunoprecipitated bands. These bands represent integrin subunits and are identified in order of increasing molecular mass as β3, pre-β1, β1, α_{vn}, and α_{fn}.

trophoretically under nonreducing conditions. Anti–R140, raised against rat Fn receptor, and anti–GP3, raised against band 3 (the β1 subunit of the chicken CSAT complex), both immunoprecipitated a β1 subunit of 117 kD and an associated α subunit of 159 kD (Fig. 2, lanes *b* and *c*). On the other hand R838, raised against human endothelial IIIa-like protein, immunoprecipitated a β3 subunit of 86 kD and an associated α subunit of 147 kD (Fig. 2, lane *d*). Nonimmune rabbit serum was nonreactive (Fig. 2, lane *a*).

Immunoprecipitation with anti–integrin antibodies from [^{35}S]methionine metabolically labeled BAEC lysates also demonstrated these integrin subunits. Specifically, anti–R140 (Fig. 2, lanes *b* and *e*) immunoprecipitated both the β1/α (117/159 kD) complex and the β3/α (86/147 kD) complex. Such cross-reactivity between β1 and β3 integrins in metabolically labeled lysates may reflect homologous epitopes that become cryptic during processing and transport of the molecules to functional cell surface complexes and is seen

in other systems as well (Albelda et al., 1989). Anti–R140 also immunoprecipitated a 101-kD band further analyzed by immunoblot.

β chain integrin subunits of molecular masses consistent with those identified by immunoprecipitation were observed on immunoblots. When probed against BAEC lysates, antibody to β1 integrin synthetic peptide primary sequence (anti–β1/10P) immunoblotted two protein species with apparent molecular masses of 101 and 117 kD under nonreducing conditions (Fig. 3, lane *a*) and 113 and 130 kD under reducing conditions (Fig. 3, lane *b*). Similar bands were detected with other antibodies to β1 integrins such as anti–R140 as well as polyclonal antibodies to the VLA β subunit and to the chicken CSAT antigen complex (data not shown). The immunoblot analyses described above suggested that the 101- and 117-kD bands identified by anti–β1/10P (as well as the other anti–β1 subunit antibodies) shared primary sequence and were therefore interrelated and unlikely to be related to the other integrin bands. Previous reports (Akiyama and Yamada, 1987; Roberts et al., 1988; Jaspers et al., 1988) have suggested the presence of a lower molecular mass im-
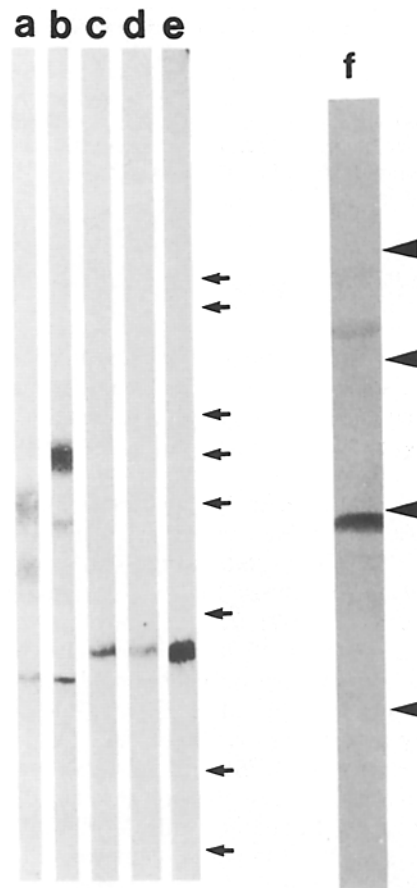

Figure 3. Immunoblot detection of BAEC integrins. BAEC lysates were electrophoresed under nonreducing conditions (lanes *a* and *c–e*) or reducing conditions (lanes *b* and *f*) and transferred to nitrocellulose. Arrows indicate molecular mass markers for 6% PAGE: (lanes *a–e*) 208, 193, 145, 132, 116, 97, 67, and 60 kD. Arrowheads indicate molecular mass markers for 10% PAGE: (lane *f*) 97, 67, 43, and 25 kD. Immunoblots were conducted with anti–β1/10P (lanes *a* and *b*), anti–GPIIIa (lane *c*), R838 (lane *d*), 22C4 (lane *e*), and anti–LB69 (P20A) (lane *f*).

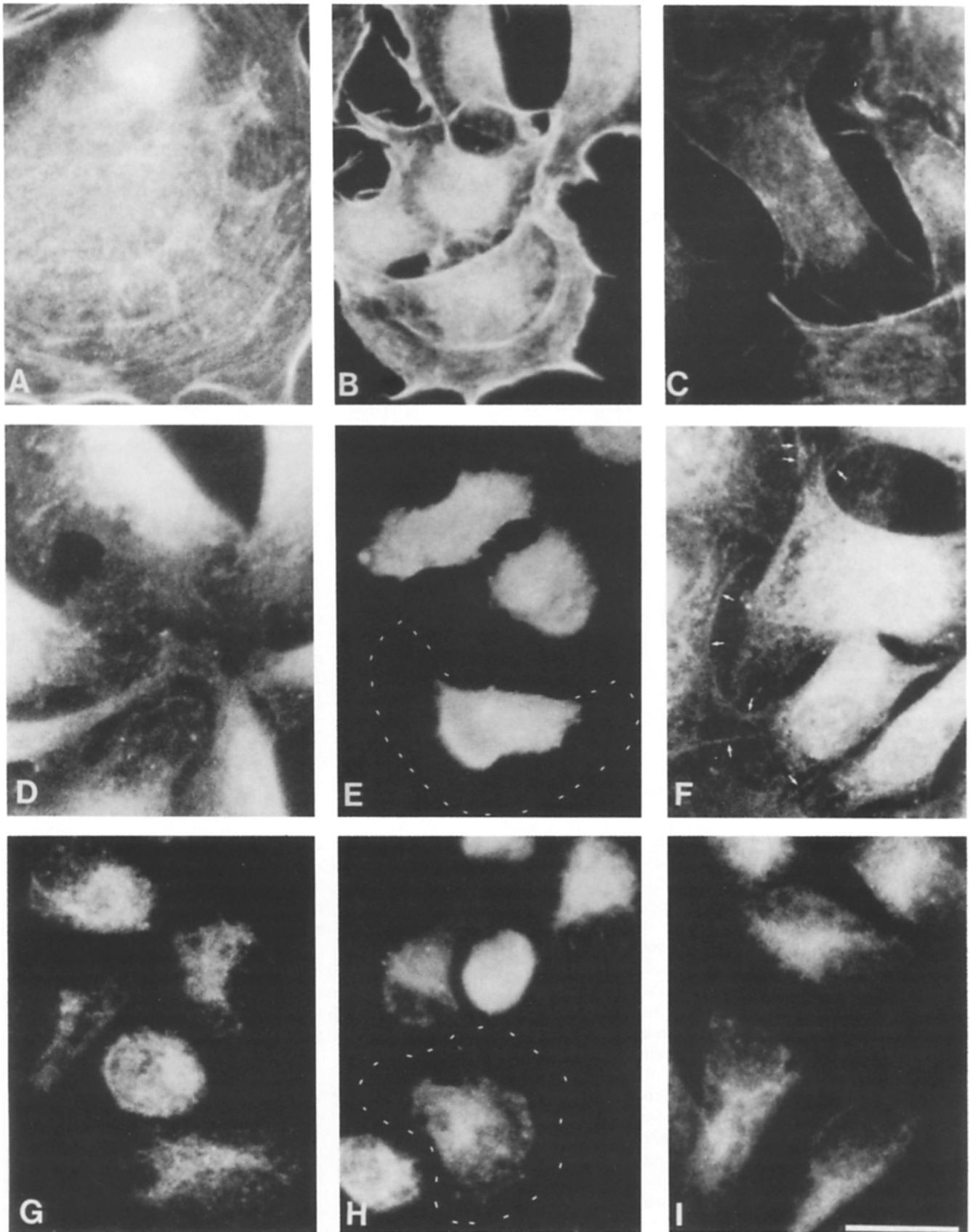*Figure 4.* Immunofluorescence localization of matrix-binding proteins in BAECs 1 h after plating. BAECs were plated on Fn (*A, D,* and *G*), Ln (*B, E,* and *H*), or Fb (*C, F,* and *I*), fixed 1 h later, and stained with anti-β1/10P (*A-C*), anti-GPIIIa (*D-F*), or anti-LB69 (*G-I*). Note the β1 integrin organization into concentric arcs when the cells are plated onto Fn (*A*) or Ln (*B*) and the β3 integrin localization to cell periphery (*arrows*) when BAECs are plated onto Fb (*F*). In all cases at this point, LB69 (*G-I*) remained in a perinuclear distribution and did not exhibit cytoplasmic process localization even in BAECs plated on Ln (*I*). Dashed lines in *E* and *H* denote cell borders of these representative cells. Bar, 5 µm.

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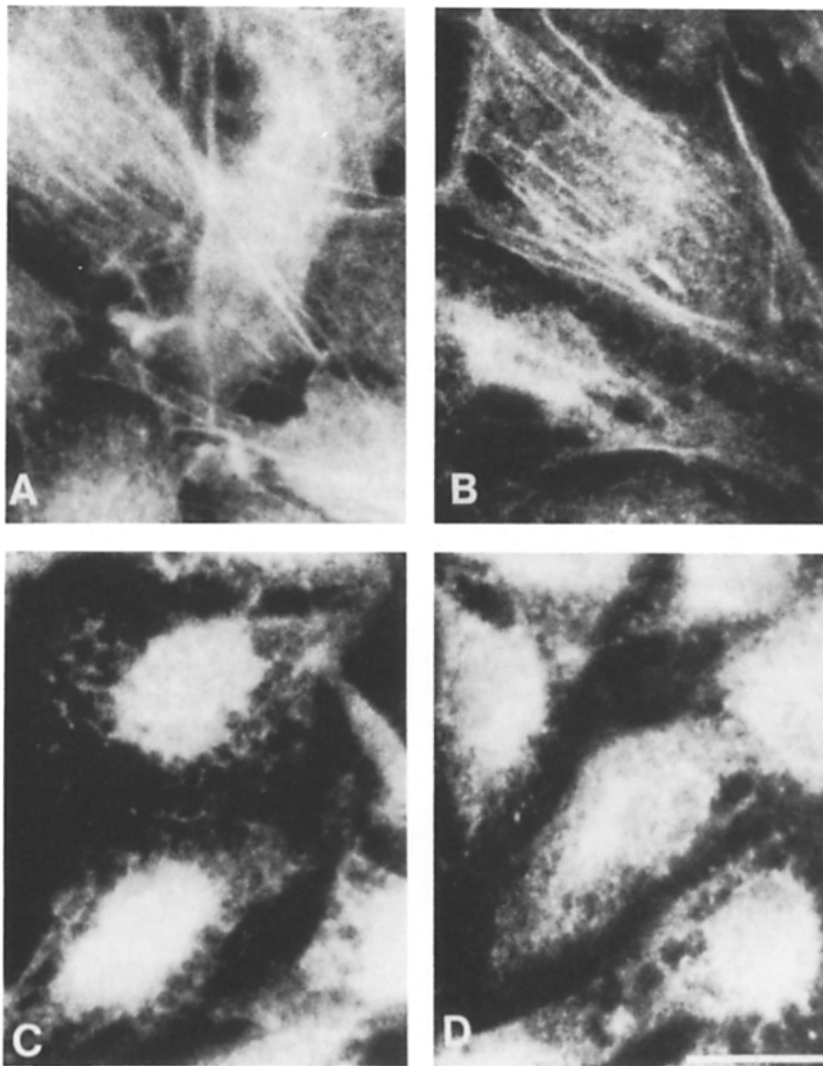
Figure 5. Immunofluorescence localization of matrix-binding proteins in confluent BAECs. BAECs plated on Fn (*A*), Ln (*B* and *D*), or Fb (*C*), grown to confluence over 3 d, fixed, and stained with anti-β1/10P (*A* and *B*), anti-GPIIIa (*C*), or anti-Ln receptor (*D*). β1 integrin organization in response to Fn (*A*) and Ln (*B*) and β3 integrin organization in response to Fb (*C*) are maintained. Note, however, the reorientation of β1 integrin fibrillar streaks (*A* and *B*). At this time point, LB69 now exhibits a diffuse punctate cytoplasmic pattern in BAECs plated on Ln (*D*). Bar, 5 μm.

mature β1 precursor form, and, given that anti–GP3 only recognizes the 117-kD band, it seemed that the 101-kD band represented such a precursor for a mature 117-kD β1 subunit protein. Pulse–chase studies (data not shown) and the absence of this β1 species on the cell surface (Fig. 2, lane *b*) were consistent with such a hypothesis.

Anti–β1/10P also recognized a smaller protein species of 80 kD independent of reduction. This band may represent a nonglycosylated core protein (as proposed by Jaspers et al., 1988), and this molecular mass is in agreement with that predicted by primary sequence deduced from cDNA clones (Argraves et al., 1987).

Antibodies to β3 integrins (anti–GPIIIa, R838, and 22C4) all immunoblotted a single band with an apparent molecular mass of 86 kD under nonreducing conditions (Fig. 3, lanes *c–e*). These antibodies were unable to recognize any material under reducing conditions.

No proteins were detected (data not shown) with monoclonal antibodies specific for β2 class integrins (anti–p150,95 and anti–LFA-1 α subunit).

In Yannariello-Brown et al. (1988), anti–LB69 was shown to immunoblot a 69-kD band. In this study we have also used an antibody (P2A) to a synthetic peptide comprising residues 263–282, which immunoblots from whole cell lysates not only the mature 69-kD protein but also its cytoplasmic 37-kD precursor (Wewer et al., 1987; Rao et al., 1989; Castronovo, V., A. P. Claysmith, H. C. Krutzsch, and M. E. Sobel, manuscript submitted for publication) as illustrated here on 10% PAGE (Fig. 3, lane *f*). This particular antibody appears to stain the 37-kD band more intensely than the 69-kD band. However, interpretation of band intensity is difficult in Western blotting with anti–peptide probes due to possible variability in epitope exposure in the two proteins bound to the membrane.

Extracellular Matrix Alters BAEC Integrin and Nonintegrin Binding Protein Organization in Static Cultures

The above described peptide and antibody studies suggested an RGD/integrin-dependent BAEC attachment to Ln, Fn, and Fb. However, the presence of immunoreactive LB69 as well as previous demonstration of cell surface LB69 (Yannariello-Brown et al., 1988) also suggested nonintegrin-mediated interactions with Ln. Therefore, immunofluorescence studies of cultured BAECs with antibodies to these matrix-binding proteins were conducted to assess matrix-

driven integrin and nonintegrin binding protein organization. Anti-β1/10P was used to assess β1 integrin organization, and anti-GPIIIa β3 integrin organization in vitro, and both antibodies were shown to bind their antigens in endothelial and smooth muscle cells in bovine aorta in situ (data not shown). Immunofluorescence studies of cultured BAECs initially were conducted at 1 h after plating to assess the matrix-binding protein organization over the same time interval as the above described attachment assays. During this 1-h interval, staining for matrix components, Fn, Fb, and Ln, revealed stored intracellular material in a perinuclear halo but the absence of matrix protein deposited extracellularly. Thus, the dominant matrix protein remained the one initially coated on the dish.

At 1 h, staining with anti-β1/10P revealed that when BAECs were plated on Fn or Ln, the β1 integrins organized into a linear stress fiber–type pattern in concentric arcs which were at or parallel to the ruffled border (Fig. 4, A and B). Anti-β1/10P only weakly stained BAECs plated on Fb (Fig. 4 C). On the other hand, staining with anti-GPIIIa for β3 integrin demonstrated a lacy, interrupted linear organization of β3 integrins, prominent at the borders of cell processes when cells were plated on Fb (Fig. 4 F). BAECs adherent to Fn did exhibit modest diffuse staining of cytoplasmic processes (Fig. 4 D). However, when the BAECs

were plated on Ln, β3 integrins appeared to be localized in a Golgi-like perinuclear halo, and no cytoplasmic process staining was apparent (Fig. 4 E). These dramatic differences in staining intensity appear to be due to changes in integrin organization rather than differences in amount expressed on the cell surfaces as cell surface iodination followed by immunoprecipitation reveals similar amounts of β1 and β3 integrins on the surfaces of BAECs cultured on Fn, Ln, and Fb (Basson, C. T., and J. A. Madri, manuscript in preparation). Finally, staining with anti-LB69 demonstrated no organization of LB69 in BAECs at 1 h after plating on any of the three substrates studied (Fig. 4, G–I). At this time, LB69 appeared to be localized to a perinuclear halo even when the cells were plated on Ln (Fig. 4 H).

Localization of these matrix-binding proteins changed with time in culture. BAECs were stained with antibodies at confluence after 3 d in culture. In these confluent cultures, where de novo deposition of additional matrix proteins somewhat obscures specific patterns, anti-β1/10P staining continued to demonstrate β1 stress fiber–type organization in BAECs plated on Fn and Ln (Fig. 5, A and B). It was also apparent that on these substrates, the β1 integrin linear pattern had reoriented with time to a longitudinal direction. Staining for β3 integrins with anti-GPIIIa persisted in BAECs plated on Fb, but had reoriented to a more punctate pattern
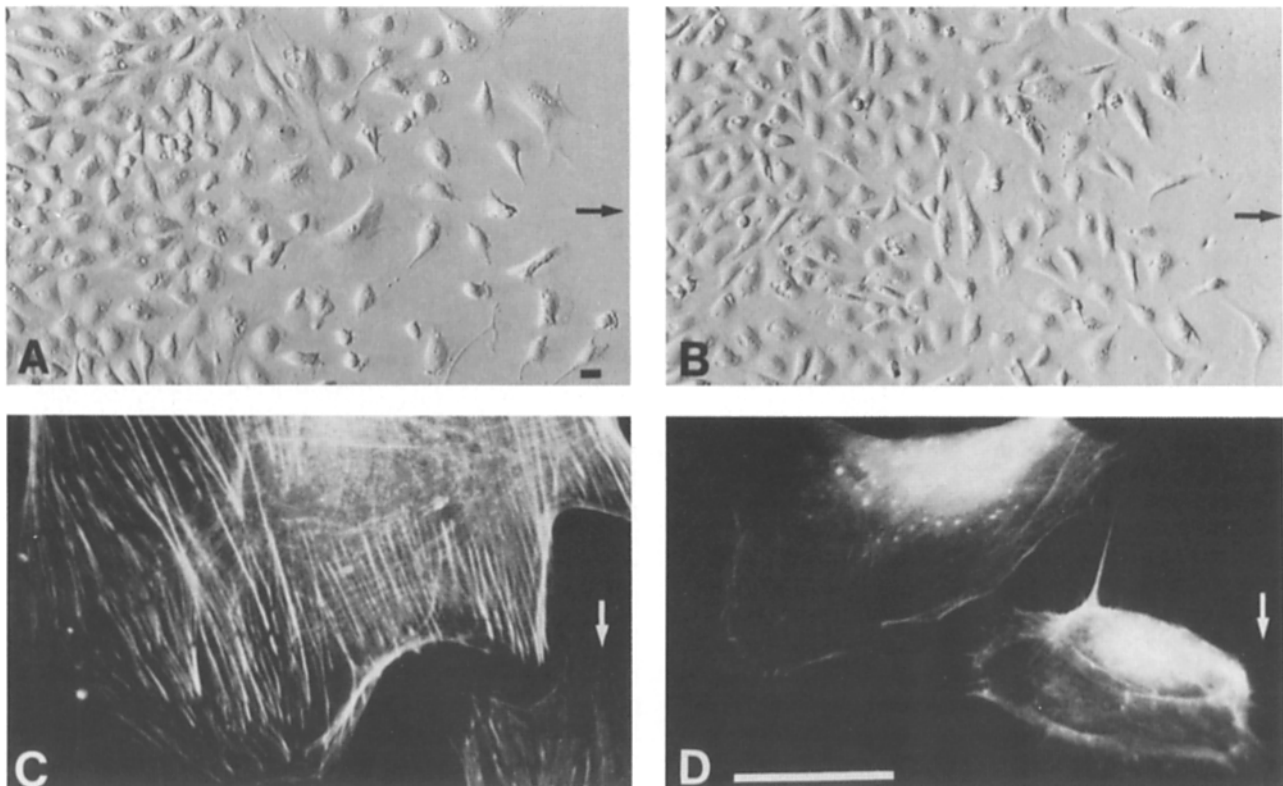

Figure 6. Hoffman interference microscopy and immunofluorescence localization of β1 integrin in BAECs migrating on Ln. BAECs were allowed to migrate over an Ln substrate in the absence (A and C) or presence (B and D) of YGRGDSP-BSA at a final peptide concentration of 5 μm. After 6 d, cultures were studied by Hoffman interference microscopy (A and B) and then fixed and stained with anti-β1/10P (C and D). C and D show cells at the leading edge of the migrating front. Arrows indicate direction of BAEC migration. Note that the cells at the migrating front of RGD-treated cultures (B and D) are smaller and more densely packed then untreated cells (A and C). Furthermore, treatment with RGD causes a dramatic disorganization (D) of β1 integrins in these cells when compared with the prominent β1 integrin fibrillar streaks of untreated migrating cells (C). Bar, 25 μm.

(Fig. 5 C). Most obvious, at this time point, was the increased diffuse cytoplasmic staining for LB69 in BAECs grown to confluence on Ln (Fig. 5 D) as compared with those newly plated on Ln (Fig. 4 H).

RGD Peptides Modulate BAEC Migration on Ln

Further studies investigated the spatiotemporal segregation of Ln-binding proteins during BAEC migration over Ln in a 6-d radial migration assay (Madri et al., 1988). After 6 d, migrating cultures were fixed and stained, areas were measured with a digitizing tablet, and daily migration rates were calculated. BAEC migration rates over Ln were clearly decreased by the addition of 5 μm YGRGDSP from 36 $\pm$ 1 to 23 $\pm$ 1 mm²/d ($n = 5$). YGRGDSP-treated and untreated BAECs migrating on Ln were further studied by Hoffman interference microscopy and immunofluorescence microscopy to localize matrix-binding proteins (Fig. 6). The cells at the migrating front of RGD-treated cultures (Fig. 7 B) were qualitatively more densely packed than those of untreated cultures (Fig. 6 A). Staining with anti–β1/10P in untreated control migrating front BAECs reveals that the β1 integrins are organized into a prominent stress fiber–like pattern (similar to the fibrillar streaks visualized by Kelly et al. [1987]

in fibroblasts with antibodies to smooth muscle integrins) oriented parallel to the axis of migration (Fig. 6 C). By contrast, when these cultures are treated with YGRGDSP, the migrating front cells exhibit a striking loss of this staining pattern. In this instance, the bulk of the β1 integrin is localized in storage granules in a perinuclear halo, and what faint linear staining exists is parallel to the ruffled border (Fig. 6 D). Regardless of the presence or absence of YGRGDSP, β3 integrins, visualized by immunofluorescence with anti–GPIIIa and not expected to bind Ln, remained localized in a perinuclear halo of storage granules (Fig. 7 A).

However, in such BAECs migrating over Ln, LB69 did exhibit a marked cytoplasmic organizational pattern that was unaltered by the presence of YGRGDSP. As seen in Fig. 7 B, LB69 organized into delicate lacy dotted linear patterns that were distributed throughout the cytoplasm of the cell. Thus, like static confluent BAECs, BAECs migrating on Ln for extended time periods displayed more intensely organized LB69 patterns than the newly plated BAECs.

Examination of β1 integrin and LB69 staining pattern in migrating BAECs with confocal microscopy revealed that both proteins were localized in basolateral domains (Fig. 8). LB69 organization insensitivity to RGD peptide treatment
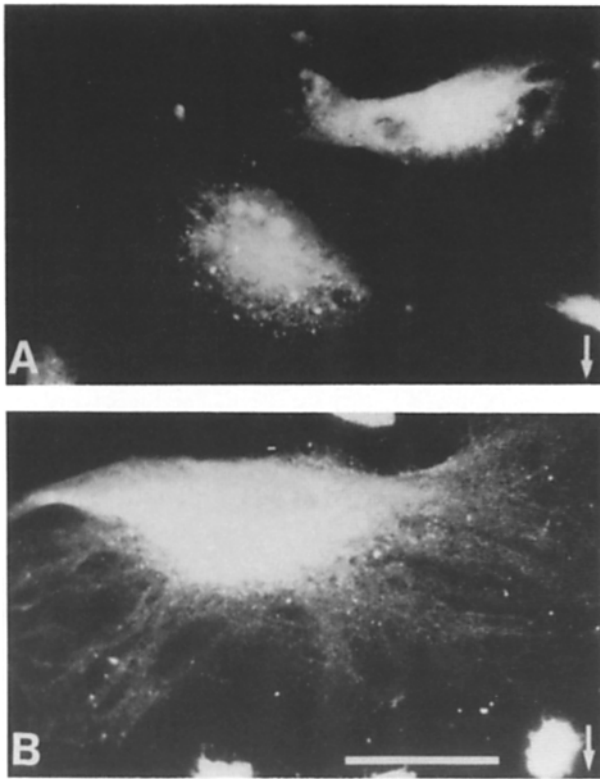

Figure 7. Immunofluorescence localization of β3 integrins and LB69 in BAECs migrating on Ln. Migrating BAEC cultures on Ln were established as in Fig. 6 in the presence or absence of YGRGDSP-BSA and processed for immunofluorescence staining with anti-GPIIIa (A) or anti-LB69 (B). No change in staining pattern was seen upon addition of RGD peptide, and cells from the leading edge of untreated migrations are shown here. Arrows indicate direction of migration. The perinuclear localization of β3 integrins (A) is consistent with inability to bind Ln. Most notable is the lacy linear cytoplasmic distribution of nonintegrin LB69 in migrating BAECs (B). Bar, 25 μm.
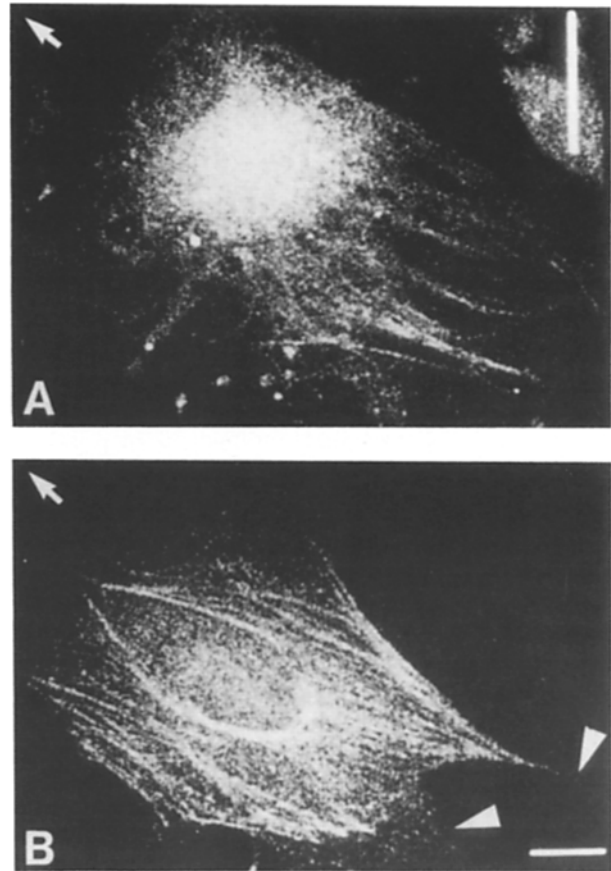
Figure 8. Confocal immunofluorescent microscopy localization of β1 integrins and LB69 in basal domains of BAECs migrating on Ln. BAEC cultures migrating over an Ln substrate were stained with anti-LB69 (A) or anti-β1/10P (B). z-sectioning analysis on a confocal imaging system was used to determine the matrix-binding protein distribution in basal domains. Arrowheads indicate cell filopodia. Bars, 25 μm.

was also evident in these domains, and the LB69 linear arrays were oriented with respect to the direction of migration (Fig. 8 A). Interestingly, the β1 integrin streaked paralleling the migration axis in the basal planes of the cells were absent from trailing filopodia, present in leading lamellopodia, and most prominent in the cell body (Fig. 8 B).

Relative Contributions of Integrins and LB69 to BAEC Attachment and Spreading on Ln

Given such morphologic evidence for matrix-binding protein spatiotemporal segregation in static and motile BAECs, we sought to assess the relative contributions of integrin and nonintegrin Ln-binding proteins in BAEC adhesion and spreading on an Ln substratum. BAEC integrin binding to Ln is shown here to be RGD dependent, presumably through an Ln A chain sequence. Others have proposed the Ln B1 chain sequence YIGSR for the LB69 ligand, and, in certain cell systems, YIGSR-NH₂ peptides can inhibit LB69 binding to Ln (Graf et al., 1987a,b; Iwamoto et al., 1987; Kubota et al., 1988).

A comparison of the effects of 1- and 2-mM doses of YGR-GDSP, YGRGESP, and YIGSR-NH₂ peptides on BAEC initial (1 h) attachment to Ln was conducted as well as a study of the effects of 2-mM doses of YGRGDSP and YIGSR-NH₂ on BAEC spreading (Fig. 9 A). As previously noted, YGRGESP at either of these doses had no effect on BAEC attachment to Ln, while YGRGDSP at such doses markedly inhibited initial BAEC attachment to Ln. However, at either 1- or 2-mM doses the putative LB69 binding peptide YIGSR-NH₂ was completely unable to alter initial cell attachment to Ln. (The addition of 1 mM YIGSR-NH₂ concomitant with 1 mM YGRGDSP did not significantly augment the effects of the RGD peptide.) However, 2-mM doses of either YGRGDSP or YIGSR-NH₂ inhibited BAEC spreading on Ln, with the numbers of rounded cells increased ~2.5-fold in either case. Furthermore, as shown in Fig. 9 A, the mean cell size was dramatically decreased by YIGSR-NH₂ peptide as well as YGRGDSP.

To further confirm that such differential effects on BAEC attachment and spreading were specific for the integrin and LB69 proteins, similar studies were undertaken with antibodies to these proteins as well as to Ln itself (Fig. 9 B). While antibodies to Ln matrix protein and to integrin (anti-GP140) were both able to inhibit BAEC attachment to Ln, antibody to nonintegrin LB69 was completely unable to inhibit attachment. Nevertheless, both antibody to integrin as well as antibody to LB69 significantly inhibited spreading. Nonimmune sera and antisera directed against human type III collagen had no effect on attachment or spreading (data not shown). Thus, functional studies with both antibodies and peptides confirmed roles for integrins and nonintegrins in adhesive events.

Discussion

Large vessel endothelial cells interact in vivo with a variety of extracellular matrix proteins, and differential expression of such proteins in the vessel wall correlates with endothelial cell response to injury (Madri et al., 1988). Our previous studies have demonstrated the presence of an Ln-binding protein LB69 on the surface of BAECs (Yannariello-Brown et al., 1988). Investigators have also documented integrin
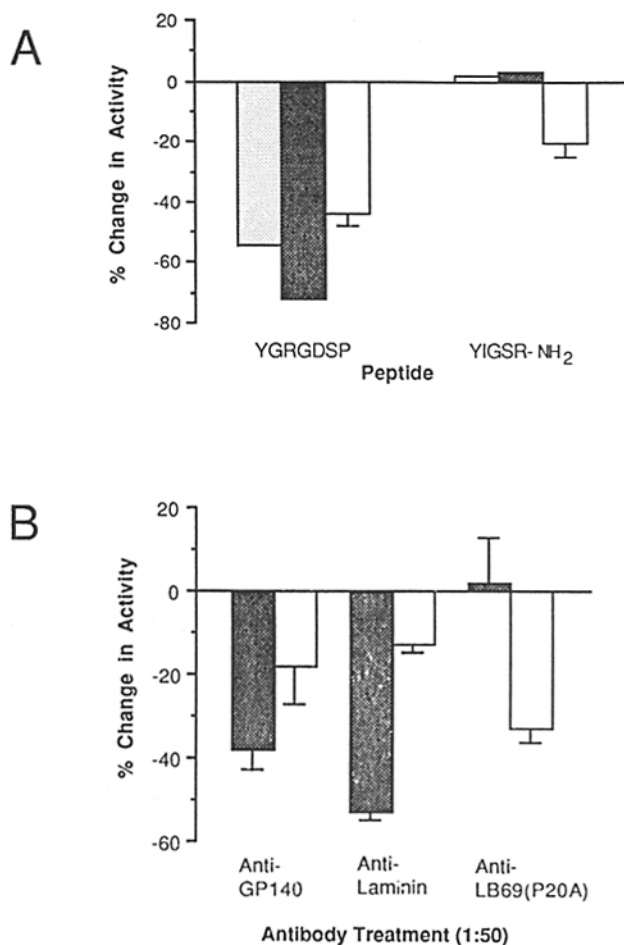
Figure 9. Comparison of integrin and LB69 contributions to BAEC attachment and spreading on Ln. BAECs were plated on an Ln substrate in the presence of either (*A*) synthetic peptides YGRGDSP and YIGSR-NH₂ or (*B*) antibodies to integrin (anti-GP140), Ln, and LB69 (anti-LB69 [P20A]). *A* presents the effects of peptides at 1-mM (*light shaded bars*) and 2-mM (*dark shaded bars*) doses on BAEC attachment after 1 h and at 2-mM doses on BAEC size (*open bars*) after 6 h. *B* presents the effects of heat-inactivated antibodies at a 1:50 dilution on BAEC attachment (*shaded bars*) after 1 h or the fraction of spread BAECs (*open bars*) at 6 h.

matrix-binding protein expression by other endothelial cell types (Dejana et al., 1988a; Cheresh, 1987), and a protein homologous to platelet GPIIIa has been found in BAECs as well (Fitzgerald et al., 1985). Therefore, BAECs are likely to interact with extracellular matrix proteins via many cellular binding proteins, each of which may have more than one matrix protein ligand. Furthermore, each matrix protein may have multiple ligand domains for several different cellular binding proteins. In this study, we identify some of the complex array of BAEC matrix-binding proteins. We then further contrast differing roles of RGD-dependent and -independent BAEC-matrix interactions in BAEC adhesive events.

We now present evidence for an integrin-dependent BAEC interaction with extracellular matrix. BAEC attachment to Ln, Fn, and Fb are all inhibited by soluble peptides containing the RGD sequence. We have observed similar differential sensitivities to RGD peptides by human iliac vein endothelial

cell adhesion (Albelda et al., 1989) to all these molecules, including Ln, and others have noted RGD sensitivity of human umbilical vein endothelial cell (HUVEC) attachment to Ln as well (Sasaki et al., 1988). In contrast, Herbst et al. (1988) did not note RGD dependence of BAEC attachment to Ln. However, in that study, it is possible that the relatively high amounts of Ln bound to the culture surface may have resulted in a peptide–protein stoichiometry which obscured this effect. We have shown here a clear dose dependency of RGD inhibition of BAEC attachment to Ln not only on RGD peptide concentration but also on amount of Ln bound to the dish and presented as substrate. Gehlsen et al. (1988b) have identified Ln-binding RGD-independent rat neuroglioblastoma integrin, but neuroglioblastoma mechanisms of attachment may differ from those of bovine endothelial cells.

Immunoblotting and immunoprecipitation analyses with antibodies to integrins reveal that BAECs express at least two different integrin heterodimers, $\beta 1$ and $\beta 3$ class integrins. Immunofluorescence studies of BAEC integrins reveal rapid matrix protein–specific organization within the first hour of initial cell attachment. In response to Fn or Ln substrates, the $\beta 1$ integrin molecules become arrayed in linear stress fiber–type patterns within 1 h of cell plating. Such linear organization is similar to the fibrillar streaks noted by Marcantonio and Hynes (1988) in Nil 8 hamster cells. In addition, in response to Fb substrates, and to a lesser extent Fn, $\beta 3$ integrins organize into a punctate pattern over cytoplasmic processes. Of note is the ability of LM609 mAb specific for $\beta 3$ integrin (provided by Dr. D. Cheresh, Research Institute of Scripps Clinic, La Jolla, CA) to inhibit BAEC attachment to Fb but not to Ln or Fn (Basson, C. T., and J. A. Madri, unpublished observations). Dejana et al. (1988a), Cheresh (1987), and others (Albelda et al., 1989) have observed similar matrix-driven organizational responses of HUVEC $\beta 1$ and $\beta 3$ integrins to Fn and Fb substrates.

We demonstrate that Ln substrates very specifically drive $\beta 1$ integrin organization in accord with reports in other mammalian and avian cell systems that cellular interaction with Ln may be mediated via RGD-dependent integrins (Tomaselli et al., 1988; Yamada and Kennedy, 1987; Chen et al., 1986; Horwitz et al., 1985). Precise organizational mechanisms in response to specific matrices remain to be determined and may reveal what distinguishes between $\beta 1$ integrin binding to Fn and $\beta 1$ binding to Ln.

Certainly, not only do BAECs bind to Ln through RGD-dependent $\beta 1$ integrins but they also express a nonintegrin Ln-binding protein, LB69. Immunofluorescence data presented here demonstrate clear differences in the spatial localization of BAEC integrins and LB69. In addition, we see not only distinct intracellular localizations of $\beta 1$ integrin and LB69 Ln-binding proteins but also temporal segregation of these proteins. For instance, BAEC $\beta 1$ integrins do organize rapidly within 1 h of cell plating on Ln, and, with time and at confluence, they maintain these organizational patterns even though the linear pattern of $\beta 1$ integrin, a molecule known to be associated with cytoskeletal proteins (Horwitz et al., 1986; Maher and Singer, 1988), does reorient in the cell as do actin stress fibers and other cytoskeletal elements (Pratt et al., 1984). In contrast, LB69 exhibits no cytoplasmic organization at 1 h after plating, but organization is visible at later time points such as at confluence. Such spatiotemporal segregation implies different functional roles in

adhesion of RGD-dependent and -independent Ln-binding proteins. This concept finds further support in our studies demonstrating that when BAEC migration over an Ln substrate is inhibited by addition of soluble RGD peptide, $\beta 1$ integrin distribution dramatically disorganizes, while LB69 remains well localized to basolateral domains in an organized linear pattern.

Others have reported non-RGD cell binding sequences not only in Ln but also in Fn (McCarthy et al., 1986; Humphries et al., 1988; Mugnai et al., 1988; Obara et al., 1988) and Fb (Plow et al., 1984; Gartner and Bennett, 1985; Santoro and Lawing, 1987) as well as RGD-independent cell surface binding proteins for these matrix molecules (Van Mourik et al., 1985; Codogno et al., 1987; Rieber et al., 1988; Humphries et al., 1988). In our investigation of the BAEC system, the contributions of RGD-dependent integrin and RGD-independent nonintegrin matrix-binding proteins to various attachment events do appear to be spatiotemporally segregated.

It is clear from the data we present here that initial BAEC adhesion to matrix substrates such as Ln occurs via an RGD-dependent integrin mechanism. Initial attachment to Ln can be blocked by both soluble RGD peptides and the calcium chelator EDTA which should disrupt the calcium-dependent (Ruoslahti and Pierschbacher, 1987) integrin binding but not the calcium-independent (Malinoff and Wicha, 1983) LB69 binding. On the other hand, YIGSR-NH$_2$ peptide has no effect on this initial attachment. While antibody to integrin inhibits BAEC attachment to Ln, neither antibody to LB69 nor YIGSR-NH$_2$ has any effect on cell attachment. (Previously described preliminary data indicating modest YIGSR potency [Yannariello-Brown et al., 1988] have proved to be artifactual due to peptide preparation impurities, and simultaneous preliminary reports of antisera to LB69 partially inhibiting BAEC attachment to Ln have been shown to be due to excessively vigorous washing, causing artifactual detachment of rounded but in fact adherent cells.) Therefore, although LB69 certainly participates in BAEC interaction with Ln since purified BAEC LB69 binds Ln in vitro, colocalizes with newly synthesized Ln in vivo, and reorganizes from apical cell surface patches to basolateral domain during migration (Yannariello-Brown et al., 1988), this nonintegrin does not contribute to initial BAEC adhesion to Ln.

On the other hand, our studies of BAEC–Ln interaction suggest that adhesion and spreading of confluent and subconfluent cultures grown on Ln for at least several hours becomes increasingly nonintegrin dependent. This matrix interaction is, at least in part, dependent on LB69 which, as mentioned above, only exhibits organization at these later time points. Moreover, during the 6 h after initial plating, antibody to LB69 and YIGSR-NH$_2$ are both able to inhibit cell spreading. Further support for nonintegrin matrix interaction is found in the observation that when RGD peptide is added to preattached motile or static BAEC cultures, inhibition of migration or rounding of cells is observed, respectively, but in either case without any cell detachment from the Ln substratum. Identical results are obtained when integrin binding is disrupted by treating these cultures with EDTA. The resistance to YGRGDSP is probably due to nonintegrin binding proteins like LB69 and not due to cell binding to newly synthesized matrix protein which may not contain active RGD sequence since these data can be obtained by intervention as early as 5 h after plating when

significant de novo matrix deposition has not yet occurred (Madri et al., 1988). Therefore, we suggest that LB69 contributes to later events in BAEC attachment such as spreading and to maintenance and to stabilization of BAEC–matrix contacts during migration.

Such segregation of BAEC integrin- and nonintegrin-dependent attachment mechanisms are not exclusive to interaction with Ln. Although nonintegrin binding proteins have not yet been conclusively identified in endothelial cells for Fn and Fb, and observation that YGRGDSP and EDTA, which can completely detach BAECs from BSA-YGRGDSP substrates, cannot detach BAECs from Fb or Fn suggests that RGD-independent binding to these matrices certainly occurs as well. Others have observed that with increased time and cell density after cell plating, RGD peptides exhibit greatly diminished capacity to detach BAECs and HUVECs from Fn (Hayman et al., 1985; Sage et al., 1989) or to detach HUVECs from Fb (Dejana et al., 1988b), although peptide potency to inhibit cell spreading and to cause cell rounding is maintained. In our own experiments, we believe that resistance to RGD peptide is unlikely to be a result of increased cell density since YGRGDSP was unable to detach BAECs in any zone of migrating cultures that comprise a range of densities from the sparse migrating front to the confluent central origin. Even in another cell system, Cardwell and Rome (1988a) have noted that although RGD peptides inhibit oligodendrocyte attachment to glial cell derived matrix, the same dose of peptides (Cardwell and Rome, 1988b) is incapable of detaching oligodendrocytes from such matrix. In fact, Cheresh (1987) has reported that LM609 mAb, specific for a functional domain of the Fb/vitronectin receptor integrin complex inhibits HUVEC attachment to Fb but does not cause detachment of preattached HUVECs from Fb.

Rees et al. (1977) have suggested that cell attachment to substrate is a multistep process of "grip and stick" that involves both strong and weak cell–matrix interactions. We therefore propose that integrin and nonintegrin extracellular matrix–binding proteins in BAECs are both temporally and spatially segregated during various attachment processes. Nonintegrin interaction with matrix, such as LB69-dependent binding, has been reported (Rao et al., 1983) to be of high affinity, 2 nM, and may thus provide the necessary stable contacts for extended periods of attachment. However, integrins which have been shown (Horwitz et al., 1985; Codogno et al., 1987) to bind their ligands with relatively low affinity (1 μM) may be important for initial cell adhesion as well as for the transient contacts made by the processes of motile large vessel endothelial cells. Future studies will further elucidate the regulation of integrin and nonintegrin expression and clarify the relative contributions of these classes of extracellular matrix–binding proteins to endothelial cell adhesion.

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