# Complex interactions between the laminin $\alpha$4 subunit and integrins regulate endothelial cell behavior *in vitro* and angiogenesis *in vivo*

**Annette M. Gonzalez*, Meredith Gonzales*, G. Scott Herron†, Usha Nagavarapu†, Susan B. Hopkinson*, Daisuke Tsuruta*, and Jonathan C. R. Jones*‡**

*Department of Cell and Molecular Biology, The Feinberg School of Medicine at Northwestern University, Chicago, IL 60611; and †Cutaneous Biology, Molecular Medicine Research Institute, Sunnyvale, CA 94085

Communicated by Laszlo Lorand, Northwestern University Medical School, Chicago, IL, October 24, 2002 (received for review May 6, 2002)

**The $\alpha$4 laminin subunit is a component of the basement membrane of blood vessels where it codistributes with the integrins $\alpha v\beta 3$, $\alpha 3\beta 1$, and $\alpha 6\beta 1$. An antibody against the G domain (residues 919-1207; $G^{919-1207}$) of the $\alpha$4 laminin subunit inhibits angiogenesis in a mouse–human chimeric model, indicating the functional importance of this domain. Additional support for the latter derives from the ability of recombinant $G^{919-1207}$ to support endothelial cell adhesion. In particular, endothelial cell adhesion to $G^{919-1207}$ is half-maximal at 1.4 nM, whereas residues 919-1018 and 1016–1207 of the G domain are poor cellular ligands. Function blocking antibodies against integrins $\alpha v\beta 3$ and $\beta 1$ and a combination of antibodies against $\alpha 3$ and $\alpha 6$ integrin subunits inhibit endothelial cell attachment to $G^{919-1207}$. Moreover, both $\alpha v\beta 3$ and $\alpha 3\beta 1$ integrin bind with high affinity to $G^{919-1207}$. Together, our studies demonstrate that the G domain of laminin $\alpha$4 chain is a specific, high affinity ligand for the $\alpha v\beta 3$ and $\alpha 3\beta 1$ integrin heterodimers and that these integrins, together with $\alpha 6\beta 1$, function cooperatively to mediate endothelial cell–$\alpha$4 laminin interaction and hence blood vessel development. We propose a model based on these data that reconcile apparent discrepancies in the recent literature with regard to the role of the $\alpha v\beta 3$ integrin in angiogenesis.**

matrix | matrix receptor | blood vessels

Laminins, heterotrimeric molecules composed of $\alpha$, $\beta$, and $\gamma$ subunits, are major components of basement membranes found in a variety of different tissue types. There are at least 14 laminin isoforms that regulate a variety of cellular functions including adhesion, migration, proliferation, cell survival, and differentiation (1–3). Although certain laminin isoforms, namely laminin 10 ($\alpha 5$, $\beta 1$, $\gamma 1$), show widespread tissue distribution, the expression of other laminin isoforms is tissue specific and tightly regulated during development (3). For example, laminin 2 ($\alpha 2$, $\beta 1$, $\gamma 1$) is expressed in the basement membrane of skeletal muscle and is believed to play an important role in basement membrane assembly and clustering of the acetylcholine receptor in the neuromuscular junction (4, 5). Laminin 5 ($\alpha 3$, $\beta 3$, $\gamma 2$) is a constituent of the basement membrane of epithelial tissue where it regulates stable adhesion of epithelium to the connective tissue (3, 6, 7). Laminins 8 ($\alpha 4$, $\beta 1$, $\gamma 1$) and 9 ($\alpha 4$, $\beta 2$, $\gamma 1$) are expressed by endothelial and smooth muscle cells, but their functions *in vivo* remain unclear (1, 8).

Compared with $\alpha 1$, $\alpha 2$, and $\alpha 5$, the $\alpha 4$ subunit present in laminins 8 and 9 contains a truncated N terminus (8–10). In this regard, it is similar to the $\alpha 3$ subunit present in laminins 5, 6, and 7. Like all other known $\alpha$ subunits, the $\alpha 4$ laminin subunit possesses a large C-terminal G domain, consisting of five structurally and functionally distinct regions ($G_1$–$G_5$; refs. 1 and 10–13). Expression of the $\alpha 4$ laminin subunit is restricted to certain tissues. It is found in vascular endothelial basement membranes of brain, muscle, and bone marrow and the perineurium of peripheral nerves, heart, developing skeletal muscle, and developing kidney (8, 9, 13–15). Indeed, the expression of $\alpha 4$ laminin protein has been used as a marker of the vascularity of certain types of tumors (8, 16).

Recent data indicate that the integrin heterodimers $\alpha 3\beta 1$ and $\alpha 6\beta 1$ may function as cell-surface adhesion receptors for $\alpha 4$-containing laminins (17). Studies from our lab have implicated the $\alpha v\beta 3$ integrin in endothelial cell adhesion to laminins containing an $\alpha 4$ subunit. Specifically, the $\alpha 4$ laminin subunit and $\alpha v\beta 3$ integrin codistribute in focal contact structures in endothelial cells (18). Moreover, antibodies against the $\alpha v\beta 3$ integrin inhibit endothelial cell adhesion to a G domain fragment of the $\alpha 4$ laminin subunit (18). However, these studies fail to address which integrins directly bind the $\alpha 4$ laminin subunit. Thus, the goal of our experiments was to determine integrin partners of the $\alpha 4$ laminin subunit and assess functions for the $\alpha 4$ laminin subunit in endothelial cells *in vivo* and *in vitro*. Here, we show that both the $\alpha 3\beta 1$ and the $\alpha v\beta 3$ integrin can bind the G domain of laminin $\alpha 4$ subunit with high affinity. Moreover, we detail complex integrin interactions with the $\alpha 4$ laminin and provide evidence that the $\alpha 4$ laminin subunit is involved in blood vessel development in an *in vivo* model.

## Materials and Methods

**Cell Culture.** Immortalized human bone marrow endothelial cells (TrHBMEC) were kindly provided by Babette Weksler (Cornell Medical School, New York) and Denise Paulin (Universite Paris VII and Institute Pasteur, Paris) (19). These were derived by immortalizing human bone marrow endothelial cells with a construct encoding the large T antigen of SV40 under the control of a truncated human vimentin gene promoter (19). The transformed cell line retained all of the characteristics of the untransformed cell line including expression of cell-surface markers such as von Willebrand factor, P-selectin, CD31, CD34, CD44, and intercellular adhesion molecule 2 (19). TrHBMEC were maintained in DMEM containing 2 mM L-glutamine, 10% FBS, and 1× RPMI vitamins. Human umbilical vascular endothelial cells (HUVEC) were a kind gift of William Schnapper (Northwestern University). Cells were maintained in endothelial cell growth medium containing 20% FBS and 1× supplement mix (Promo Cell, Heidelberg).

**Antibodies.** Mouse monoclonal antibodies against the $\alpha v\beta 3$ integrin heterodimer (LM609), $\alpha 3$ integrin subunit (P1B5), $\beta 1$-integrin subunit (6S6), $\alpha 3\beta 1$ integrin heterodimer (MKID2), and a $\beta 3$ integrin rabbit anti-serum (AB1932) were obtained from Chemicon. The rat monoclonal $\alpha 6$ integrin antibody (GoH3) was purchased from Beckman Coulter. 2A3, a function-blocking antibody against the G domain of $\alpha 4$ laminin was described (18). A monoclonal antibody against human collagen

Abbreviations: TrHBMEC, immortalized human bone marrow endothelial cells; HUVEC, human umbilical vascular endothelial cells.

‡To whom correspondence should be addressed. E-mail: j-jones3@northwestern.edu.

type IV was obtained from Sigma. An antiserum against von Willebrand factor was purchased from Neomarkers (Fremont, CA).

**Matrix Proteins and Integrins.** Human fibronectin and Matrigel were purchased from BD Biosciences (Bedford, MA), whereas laminin-1 was obtained from GIBCO/BRL. They were used according to each manufacturer's instructions. Laminin 5 was derived from conditioned medium of cultured epithelial cells (20). Recombinant $\alpha$4 laminin, consisting of a portion of the $G_1$ and $G_2$ subdomains (residues 919–1207; $G^{919-1207}$), was isolated from bacterial extracts as described (18). The $\alpha$4 laminin $G_1$ (residues 919–1018; $G^{919-1018}$) and $G_2$ (residues 1016–1207; $G^{1016-1207}$) fragments were produced in bacteria as follows. In brief, cDNA, generated by RT-PCR from mRNA isolated from TrHBMEC, was used as template for PCR using $\alpha$4 laminin subunit specific forward and reverse primers. Amplified product, digested with appropriate restriction enzymes, was ligated into the pET32a protein expression vector (Novagen) in frame with sequences encoding a 6 × His tag. Reading frame and sequence was verified by automated sequencing (Biotechnology Facility, Northwestern University). Vectors were transfected into the *Escherichia coli* strain BL21. The cells were induced to express laminin $\alpha$4 fusion proteins by addition of 1 mM isopropyl $\beta$-D-thiogalactoside (Fisher), and fragments were purified by column chromatography (Novagen). The purity of recombinant polypeptides was assessed by visualizing protein samples by SDS/PAGE as well as by Western blotting using a His probe, following transfer of protein to nitrocellulose (Pierce). Soluble $\alpha v\beta 3$ and $\alpha 3\beta 1$ integrin heterodimers were purchased from Chemicon. Their purity was routinely assessed by SDS/PAGE before use.

**Cell Adhesion Assay.** Approximately $1 \times 10^5$ TrHBMEC or HUVEC were plated onto uncoated or protein-coated wells of a 96-well plate (Sarstedt) and blocked with 1% BSA in PBS for 1 h at 37°C. After 1 h at 37°C, the wells were washed extensively with PBS to remove nonadhering cells, and then adherent cells were fixed in 3.7% formaldehyde in PBS for 15 min. Fixed cells were incubated at room temperature with crystal violet for 15 min and then solubilized with 1% SDS. Absorbance at 570 nm was measured with a $V_{max}$ plate reader (Molecular Devices). Values in the concentration-response curves were normalized to maximum cell attachment. The effective concentration ($EC_{50}$) is defined as the concentration of ligand that produces half-maximal cell attachment.

In certain studies, integrin antibodies and control, isotype-matched immunoglobulins were added to cell suspensions for 30 min at room temperature before the cells were plated onto substrate. In function-blocking antibody studies, values were normalized to control (100%).

**ELISAs.** Wells of 96-well nontissue culture-treated plates were coated with protein at varying concentrations for 18 h at 4°C. Each well was rinsed three times and blocked with 1% BSA in PBS for 1 h at 37°C. Soluble integrin heterodimers were diluted in binding buffer (25 mM Tris buffer/150 nM NaCl/1 mM $MgCl_2$/0.5 mM $MnCl_2$/0.05% BSA, pH 7.5) and added to each well for a final concentration of 5 ng/$\mu$l. After incubating for 90 min at 37°C, wells were rinsed three times in binding buffer, and appropriate mouse monoclonal anti-integrin antibody was added for 1 h at 37°C. Wells were then rinsed three times in PBS, and alkaline phosphatase-conjugated goat anti-mouse antibody was added to the wells for an additional 1 h at 37°C. Wells were rinsed three times in PBS, and 200 $\mu$l of substrate [*p*-nitrophenyl phosphate (PNPP, Sigma) diluted in ELISA buffer to a final concentration of 1 mg/ml] was added per well. Absorbance at 405 nm was measured with a $V_{max}$ plate reader (Molecular Devices). Nonspecific binding was determined by the addition of 10 mM EDTA to binding buffer. Specific binding was obtained by subtracting nonspecific binding from total binding (total binding − nonspecific binding). In saturation binding studies, the dissociation constant ($K_d$) corresponds to the concentration of ligand that produces half-maximal specific binding. In competition binding studies, the inhibitory concentration ($IC_{50}$) is defined as the concentration of competitor that blocks 50% of specific binding. All curves were fitted with nonlinear regression by using GRAPHPAD PRISM (v. 3.00).

**Immunofluorescence Microscopy.** Human renal carcinoma tissue was frozen in Tissue-Tek O.C.T. compound (Miles), and consecutive frozen sections of 6-$\mu$m thickness were prepared by using a Tissue-Tek Cryostat at −20°C. Sections were placed on slides, extracted in acetone at −20°C for 5 min, and then air-dried. Matrigel implants were fixed in 10% buffered formalin, embedded in paraffin, and sectioned. Sections were deparaffinized, and antigens were retrieved in 10 mM citric acid (pH 6.0) by microwaving twice for 7 min. Tissue sections were incubated with primary antibodies, diluted in PBS at 37°C in a humid chamber for at least 1 h, washed three times in PBS, and then incubated with the appropriate mix of fluorochrome-conjugated secondary antibodies for an additional 1 h at 37°C. Stained specimens were viewed by using a Zeiss LSM510 laser-scanning confocal microscope or Zeiss Axioskop microscope.

**SDS/PAGE and Western Blotting.** Matrix proteins and integrins were separated on 7.5–12% SDS-polyacrylamide gels following standard procedures (21). Gels were stained or separated proteins were transferred to nitrocellulose, which was subsequently processed for Western blotting as previously described (21–23).

***In Vivo* Angiogenesis Assay.** Approximately $1 \times 10^6$ human dermal microvascular endothelial cells were mixed with 0.5 ml of Matrigel on ice in the presence of either antibody 2A3 or control IgM, and the mixture was implanted into the ventral midline thoracic tissue of a mouse following procedures outlined in ref. 24. At 7 days, the implants were removed, fixed and processed for immunofluorescence microscopy as above. Separate ×10 fields per tissue were taken, and the number of annular structures was counted and averaged.

## Results

**The $\alpha$4 Laminin and Angiogenesis.** Our previous work has implicated a role for the $\alpha$4 laminin subunit in endothelial cell branching morphogenesis (18). Such morphogenesis is a component of blood vessel development (25). Thus, we assessed whether the $\alpha$4 laminin subunit is involved in blood vessel formation assembly *in vivo* by using a mouse–human chimeric model in which human dermal microvascular endothelial cells are injected into severe combined immunodeficient mice in Matrigel (24). After 7 days, the human cells assembled into blood vessels that can be identified and quantified by using a marker of basement membrane assembly, namely an antibody probe specific for human collagen type IV (Fig. 1 *A* and *B*) and an antiserum specific for von Willebrand factor, an endothelial cell marker (Fig. 1 *C* and *D*). In our studies, we evaluated blood vessel development in this model system under conditions where an antibody (2A3) against the $\alpha$4 laminin subunit or an isotype-matched control IgM was added to the cell–Matrigel mix before injection into the severe combined immunodeficient mice. As can be seen in Fig. 1 *A–D*, there is a significant decrease in both collagen IV antibody and von Willebrand factor staining in the 2A3 antibody-treated samples compared with samples treated with control IgM. Quantification of these results is shown in Fig. 1*E*. These data provide direct evidence that the $\alpha$4 laminin subunit, in particular the 2A3 epitope, is involved in angiogen-

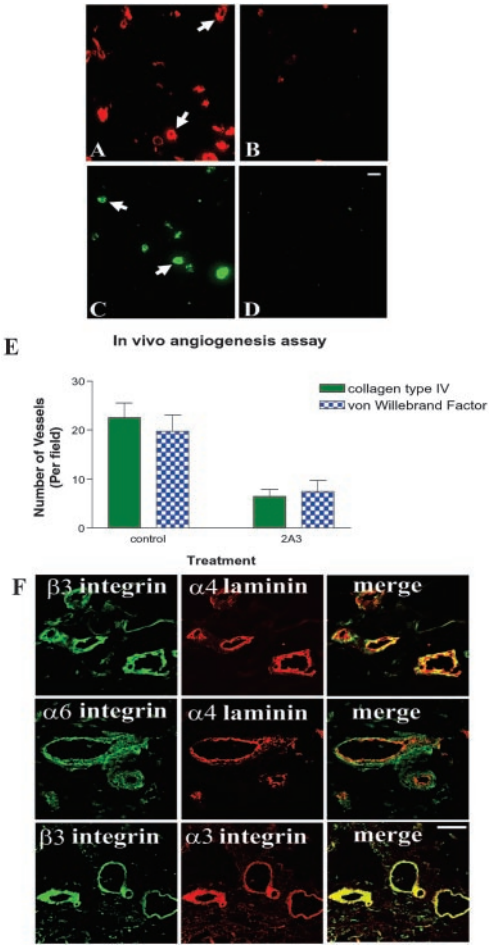


**Fig. 1.** Antibody 2A3 against $\alpha$4 laminin inhibits angiogenesis *in vivo*. Human endothelial cells were mixed with 0.5 ml of Matrigel in the presence of 125 $\mu$g/ml control IgM (*A* and *C*) or 125 $\mu$g/ml antibody 2A3 (*B* and *D*). The cell-matrix mix was then injected in the ventral midline thoracic tissue of severe combined immunodeficient mice. After 7 days, implants were removed, fixed, and prepared for immunofluorescence microscopy. Samples were stained with either anti-human type IV collagen antibody (*A* and *B*) or an antiserum against von Willebrand factor (*C* and *D*). Type IV collagen and von Willebrand factor staining appears in an annular and linear organization in *A* and *C* (arrows). (*E*) The number of vascular structures observed in the specimens shown in *A*–*D* were quantified. Results represent mean ± SD of five separate fields. (*F*) Laminin $\alpha$4 subunit and integrin subunit localization in blood vessels. Cryostat sections of human renal carcinoma tissue were prepared for double-label immunofluorescence and viewed by laser-scanning confocal microscopy. The tissue sections were incubated with a polyclonal rabbit antiserum against the integrin $\beta$3 in combination with either antibody 2A3 against the $\alpha$4 laminin subunit or antibody P1B5 against $\alpha$3 integrin as indicated. Sections were also processed by using antibody GoH3 against $\alpha$6 integrin in combination with the $\alpha$4 laminin subunit as shown. Merged versions of the red and green images are presented (*Lower Right*). Yellow color indicates overlap in staining. (Bar = 20 $\mu$m in *D*; bar = 50 $\mu$m in *F*.)

esis. Because the 2A3 epitope lies within the G domain of the $\alpha$4 laminin, we next studied the cell-surface interactions of this functionally important domain (18). We initiated our studies by first determining which integrins codistributed with the $\alpha$4 laminin subunit in the basement membrane of blood vessels.

**Endothelial Cell Integrins and the $\alpha$4 Laminin Subunit.** In cryosections of renal carcinoma tissue that possess an extensive vasculature, 2A3 antibodies generated an intense stain along the basement membrane zone of blood vessels, a site rich in $\alpha$3, $\alpha$6, and $\beta$3 subunit-containing integrins (Fig. 1*F*). Indeed, the codistribution of the $\alpha$4 laminin subunit with $\alpha$3 and $\alpha$6 integrin along the site of endothelial cell–basement membrane zone interaction is consistent with data indicating that cells interact with laminins 8 and 9 via $\alpha3\beta1$ and $\alpha6\beta1$ integrin (17). However, we showed that endothelial cells adhere to a fragment of the $\alpha$4 laminin subunit consisting of a portion of its $G_1$ and $G_2$ subdomains ($G^{919–1207}$) in an $\alpha v\beta3$ integrin-dependent manner (18). To resolve the issue of integrins involved in endothelial cell attachment to the G domain of the $\alpha$4 laminin, we undertook endothelial cell adhesion assays by using a number of $\alpha$4 G domain fragments prepared from bacterial lysates (Fig. 2*A*). To do so, we made use of bone marrow endothelial cells because these cells have been implicated in pathologically induced angiogenesis (26). Because primary bone marrow endothelial cells are not available in sufficient quantities for our studies, we used an immortalized bone marrow endothelial cell line (TrHBMEC) that shows many, if not all, of the characteristics of primary cells (19). For comparison, we also analyzed matrix adhesion of normal endothelial cells (HUVEC).

TrHBMEC or HUVEC were added to wells precoated with varying concentrations of G domain fragments comprising residues 919-1207 ($G^{919–1207}$), containing the epitope of antibody 2A3, residues 919-1018 ($G^{919–1018}$) within the $G_1$ subdomain, and residues 1016–1207 ($G^{1016–1207}$) within the $G_2$ subdomain (Fig. 2). Both TrHBMEC and HUVEC attached to residues $G^{919–1207}$ in a concentration-dependent manner, with cell binding being half-maximal ($EC_{50}$) at 1.4 and 1.5 nM, respectively (Fig. 2*B*). Endothelial cell attachment to fibronectin produced a similar concentration–response curve with an $EC_{50}$ of 1.0 nM (TrHBMEC) and 0.8 nM (HUVEC). In contrast, both $G^{919–1018}$ and $G^{1016–1207}$ fragments were poor ligands for cell attachment, and cell attachment failed to reach half-maximal even at 100 nM ligand concentration (Fig. 2*B*).

We next investigated integrin involvement in endothelial cell adhesion to $G^{919–1207}$. TrHBMEC or HUVEC in suspension were treated with various function-blocking integrin antibodies before addition to wells coated with 100 nM $G^{919–1207}$. Both TrHBMEC and HUVEC showed maximal binding to wells coated with this concentration of protein (Fig. 2*B*). Antibody LM609, which perturbs the function of the $\alpha v\beta3$ integrin, inhibited TrHBMEC adhesion to $G^{919–1207}$ by ≈70%, whereas 6S6, an integrin $\beta$1 function-blocking antibody, inhibited cell adhesion by ≈84% compared with control IgG-treated cells (Fig. 3*A*). Studies with HUVEC produced similar results (Fig. 3*A*). LM609 and 6S6 inhibited cell adhesion by 80% and 70%, respectively. The latter result is contrary to our previous report where we showed that a different $\beta$1 integrin antibody (P4C10) failed to inhibit endothelial cell adhesion to the $\alpha$4 laminin G domain (18). We have repeated our studies numerous times and consistently observe inhibition in adhesion of endothelial cells to $G^{919–1207}$ with 6S6 but little, if any, inhibition when endothelial cells are treated with P4C10. We cannot explain this anomaly. To resolve the potential role of $\beta$1 containing integrins in endothelial cell attachment to the $\alpha$4 laminin subunit, we examined the effects of antibodies that functionally inhibited $\alpha$6 and $\alpha$3 integrin, namely GoH3 and P1B5, on adhesion of endothelial cells to $G^{919–1207}$. The $\alpha$3 integrin antibody, P1B5, when used in combination with GoH3, the $\alpha$6 integrin antibody, inhibited both TrHBMEC and HUVEC adhesion by >54% (Fig. 3*A*). It should be noted that these same antibodies when used individually have minimal impact on either TrHBMEC or HUVEC cell adhesion to $G^{919–1207}$, suggesting that the function of $\alpha$3 and $\alpha$6 subunit-containing integrins is in some way coupled in endothelial cells (Fig. 3*A*). Together, these data indicate an involvement of both $\alpha6\beta1$ and $\alpha3\beta1$ in adhesion to the G domain of the $\alpha$4 laminin subunit, a finding consistent with a previous report (17). However, these data also indicate that the $\alpha v\beta3$ integrin subunit plays a role in endothelial cell–$\alpha$4 laminin subunit interaction.

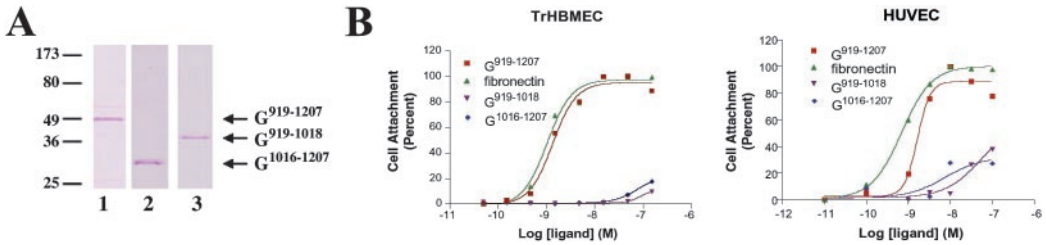


**Fig. 2.** (*A*) Gel profiles of the recombinant proteins used in these studies. Proteins purified from bacterial cell extracts were processed for SDS/PAGE. Lanes 1–3 show $G^{919–1207}$, $G^{919–1018}$, and $G^{1016–1207}$, respectively. Molecular weight standards are indicated (*Left*). (*B*) Endothelial cells (TrHBMEC or HUVEC as indicated) adhere to the G domain of the $\alpha$4 laminin subunit. Endothelial cells were added to the wells of a 96-well plate coated with varying concentrations of $G^{919–1207}$, $G^{919–1018}$, $G^{1016–1207}$, or human fibronectin as indicated. Cells were allowed to attach at 37°C for 1 h. Nonadherent cells were washed off the wells, and the remaining cells were fixed and stained with crystal violet. Absorbance was read at 570 nm. The curves are representative of three separate experiments.

One intriguing aspect of the above results is that $\alpha v\beta 3$ integrin fails to "compensate" in mediating binding to $G^{919–1207}$ when the $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrin heterodimers are functionally inhibited and vice versa (Fig. 3*A*). One possible explanation for our results is that in endothelial cells the activity of one integrin may modulate ligand binding of another via a process that is termed transmodulation (27, 28). To test this possibility, we assayed TrHBMEC and HUVEC adhesion to laminin 5, a ligand for $\alpha 3\beta 1$ but not for $\alpha v\beta 3$, in the presence of antibodies that functionally perturb either the $\alpha v\beta 3$ or $\alpha 3\beta 1$ integrin heterodimer (Fig. 3 *B* and *C*). As would be expected, TrHBMEC and HUVEC adhesion to laminin 5 was inhibited by 40% and 45%, respectively, when treated with a combination of antibodies against the $\alpha 3$ integrin subunit and $\alpha 6$ integrin subunits and by more than 80% and 60%, respectively, by antibody 6S6, a function-blocking antibody against $\beta 1$ integrin (Fig. 3*B*). However, in addition, TrHBMEC and HUVEC adhesion was also inhibited by 60% and 40%, respectively, when cells were treated with antibody LM609 against the $\alpha v\beta 3$ integrin (Fig. 3*B*). This indicates that functional perturbation of the $\alpha v\beta 3$ integrin has a transmodulating, in this case inhibitory, impact on $\alpha 3\beta 1$ integrin–laminin 5 interaction.

**Characterization of Direct Integrin Interaction with the G Domain of the $\alpha$4 Laminin Subunit.** The above data indicate a role for both the $\alpha v\beta 3$ and $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrin heterodimers in mediating endothelial cell adhesion to laminin $\alpha$4. However, they do not show whether integrins bind directly to the $\alpha$4 laminin. Hence, to study this issue, we conducted solid-phase saturation binding experiments by using a cell-free system, by using purified integrin and laminin proteins. To date, we have restricted our analyses to $\alpha v\beta 3$ and $\alpha 3\beta 1$ integrin binding to ligand because we have been unable to obtain appropriately pure $\alpha 6\beta 1$ integrin to use in our assays. Nevertheless, soluble $\alpha v\beta 3$ bound $G^{919–1207}$ in a concen-

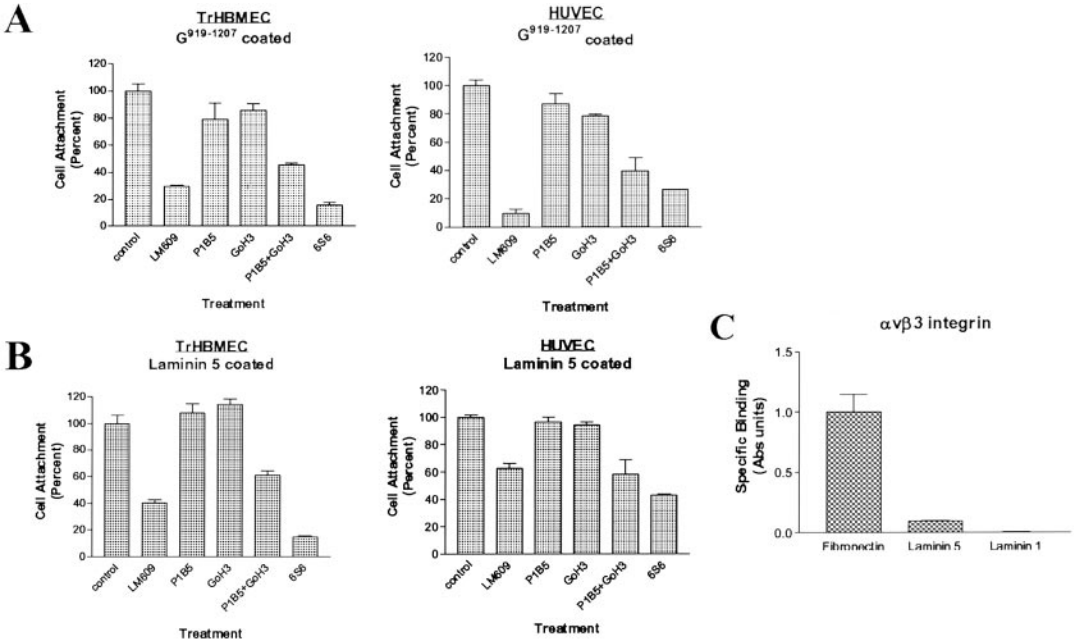


**Fig. 3.** (*A*) Cell attachment of TrHBMEC or HUVEC to $G^{919–1207}$ involves the $\alpha v\beta 3$ and $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrin heterodimers. Cells were pretreated with control IgG or function-blocking antibodies against $\alpha v\beta 3$ integrin (LM609), $\alpha 3$ integrin (P1B5), $\alpha 6$ integrin (GoH3), or a combination of P1B5 and GoH3 or $\beta 1$ integrin (6S6) for 30 min at 37°C before adding cells to wells coated with 100 nM $G^{919–1207}$ protein. LM609 was used at 25 $\mu$g/ml, whereas all other antibodies and control IgG were used at 50 $\mu$g/ml. Cell attachment was evaluated as in Fig. 2. (*B*) TrHBMEC and HUVEC adhesion to laminin 5. Endothelial cells were pretreated with the same function-blocking antibodies as in *A* for 30 min at 37°C. The attachment of the cells to wells coated with 5 $\mu$g/ml laminin 5 was evaluated after 1 h as above. (*C*) Integrin $\alpha v\beta 3$ binds directly to fibronectin but not to laminins 1 and 5. Wells of 96-well plates were coated with equal concentrations of extracellular matrix proteins (5 ng/$\mu$l). $\alpha v\beta 3$ integrin (5 ng/$\mu$l) was then added to the wells and allowed to bind for 1 h at 37°C. Integrin binding was evaluated by ELISA using an antibody against $\alpha v\beta 3$, followed by a secondary antibody conjugated to alkaline phosphatase. Absorbance was measured at 405 nm. Values in bar graphs are expressed as means ± SD of three trials.

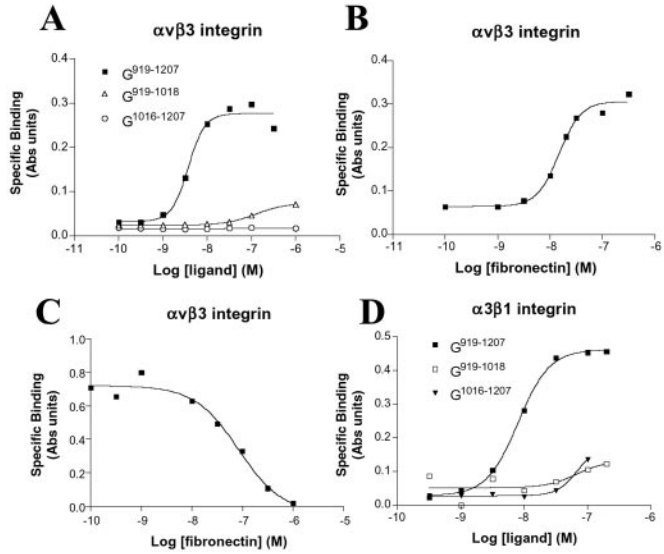


**Fig. 4.** (*A* and *B*) Integrin $\alpha v\beta 3$ binds directly to the $\alpha 4$ G domain and fibronectin with high affinity. Wells of a 96-well plate were coated with varying concentrations of $G^{919-1207}$, $G^{919-1018}$, and $G^{1016-1207}$ (*A*) or fibronectin (*B*). $\alpha v\beta 3$ integrin (5 ng/$\mu$l) was then added to the wells and allowed to bind for 1 h at 37°C. Integrin binding was evaluated by ELISA using an antibody against $\alpha v\beta 3$, followed by a secondary antibody conjugated to alkaline phosphatase. (*C*) Competition binding curve. Soluble $\alpha v\beta 3$ (5 ng/$\mu$l) was added to wells coated with $G^{919-1207}$ in the presence of increasing concentrations of fibronectin at 37°C. Integrin binding was assayed as in *A* and *B* after 1 h. (*D*) $\alpha 3\beta 1$ integrin was added to wells coated with varying concentrations of $G^{919-1207}$, $G^{919-1018}$, and $G^{1016-1207}$ and allowed to bind for 1 h at 37°C. Integrin binding was evaluated by ELISA using MKID2, an antibody against the $\alpha 3\beta 1$ integrin heterodimer, followed by alkaline phosphatase-conjugated secondary antibody. In all studies, absorbance was measured at 405 nm. Each of the graphs is representative of at least three separate experiments.

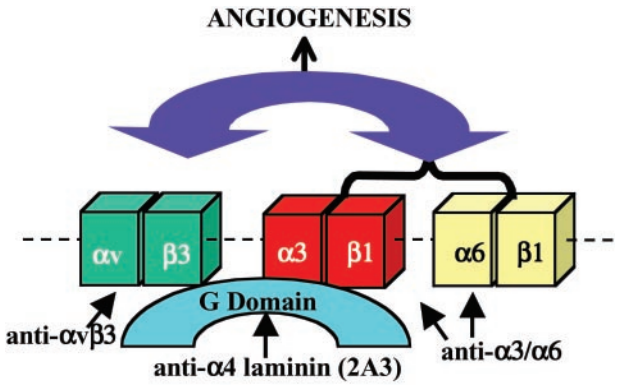


**Fig. 5.** Diagram showing a scheme in which there is crosstalk among integrins in endothelial cell adhesion to the G domain of the $\alpha 4$ laminin subunit. In the model, we show that both the $\alpha v\beta 3$ and $\alpha 3\beta 1$ integrins interact directly with the G domain. Endothelial cell adhesion to the G domain can be inhibited by G domain antibodies (2A3), by antibodies against the $\alpha v\beta 3$ integrin, by antibodies against the $\beta 1$ integrin, or by a combination of antibodies against the $\alpha 3$ and $\alpha 6$ integrin subunits. In the model, when $\alpha v\beta 3$ integrin function is inhibited, this also perturbs the function of $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrin heterodimers (bracketed) and vice versa. In the diagram, integrin interplay is indicated by a double-headed arrow. Moreover, angiogenesis is blocked when integrin function is perturbed. In the absence of $\alpha v\beta 3$ integrin, the $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrin heterodimers are capable of supporting angiogenesis but may do so in a relatively unregulated manner.

tration-dependent fashion, and binding is saturable (Fig. 4*A*). The observed dissociation constant ($K_d$) was 4.0 nM. This is comparable to the dissociation constant when $\alpha v\beta 3$ integrin bound fibronectin, a known ligand for this integrin heterodimer ($K_d$, 15 nM; Fig. 4*B*; ref. 29). Moreover, fibronectin was able to compete with $G^{919-1207}$ for binding to $\alpha v\beta 3$ in a concentration-dependent fashion with a measured $IC_{50}$ of 84 nM, suggesting that fibronectin and $G^{919-1207}$ bound to a similar or nearby site on the $\alpha v\beta 3$ integrin molecule (Fig. 4*C*). $\alpha 3\beta 1$ integrin also bound to $G^{919-1207}$ with a $K_d$ of 7.3 nM (Fig. 4*D*). No significant binding of $\alpha 3\beta 1$ or $\alpha v\beta 3$ to $G^{919-1207}$ was detected in the presence of 10 mM EDTA (data not shown). Furthermore, $\alpha v\beta 3$ and $\alpha 3\beta 1$ integrin bound poorly to both $G^{919-1018}$ and $G^{1016-1207}$ and laminin-1 (Figs. 3*C* and 4 *A* and *D*; $\alpha 3\beta 1$ integrin binding data not shown in Fig. 3*C*).

## Discussion

In this study, we have characterized a complex integrin-binding domain within the $\alpha 4$ laminin subunit. During the course of our studies, we have shown that two different endothelial cell types and purified integrins adhere to a fragment of the $\alpha 4$ G domain that contains a portion of the $G_1$ and $G_2$ subdomains. Individually, neither the $G_1$ nor the $G_2$ subdomain possesses an obvious binding site for endothelial cells or its integrin heterodimers. Rather, cells and integrins show interaction with a region that seems to span both subdomains. The functional importance of the $\alpha 4$ laminin G domain is emphasized by our *in vitro* and *in vivo* studies. In the former, we have shown that antibodies against the same $G_1/G_2$ domain fragment block branching morphogenesis of endothelial cells maintained on artificial basement membrane proteins (18). In the latter, we show that the same antibody inhibits blood vessel development *in vivo*. The idea that the G domain of the $\alpha 4$ laminin subunit plays an important role in blood vessel formation as well as function extends recent studies in which the phenotype of mice lacking the $\alpha 4$ laminin subunit has been analyzed (30). These mice possess leaky blood vessels.

One intriguing aspect of this work is that various integrin heterodimers seem to act in concert in endothelial cell adhesion to the G domain of the laminin $\alpha 4$ subunit. By using cell adhesion assays, we have presented evidence that function-perturbing antibodies against the $\alpha v\beta 3$ integrin, $\beta 1$ integrin subunit or a mix of antibodies against the $\alpha 3$ and $\alpha 6$ integrin subunit inhibit endothelial cell adhesion to the $\alpha 4$ laminin G domain. This result led us to assess which integrins are able to bind directly to the $\alpha 4$ G domain. Our data reveal that both $\alpha v\beta 3$ and $\alpha 3\beta 1$ integrin bind the $\alpha 4$ G domain with high affinity. This finding perplexed us. If both are capable of direct interaction with ligand, one might assume that when one is functionally inhibited then the other should be able to compensate and mediate cell-ligand binding alone. However, this is not the case. The most obvious explanation for this finding is that integrin heterodimers show a complex functional interaction when endothelial cells adhere to the $\alpha 4$ laminin subunit G domain such that when the $\alpha v\beta 3$ integrin is blocked by antibody, then there is a concomitant block in ligand binding of the $\alpha 3\beta 1$ and $\alpha 6\beta 1$ heterodimers. Likewise, blocking both $\alpha 3\beta 1$ and $\alpha 6\beta 1$ has a negative impact on the ability of $\alpha v\beta 3$ integrin heterodimer to bind its ligand. This model of integrin cooperativity is shown diagrammatically (Fig. 5). Moreover, we have provided some experimental evidence for our model because endothelial adhesion to laminin-5, a ligand for the $\alpha 3\beta 1$ integrin but not the $\alpha v\beta 3$ integrin, is inhibited by antibody LM609 directed against the $\alpha v\beta 3$ integrin heterodimer.

For a number of years, the $\alpha v\beta 3$ integrin heterodimer has been believed to play an important role in angiogenesis (29, 31, 32). This notion has recently been questioned because a number of groups have shown that mice lacking either the $\alpha v\beta 3$ integrin or both $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins develop normally (33–35). Moreover, mice lacking both $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins develop more extensive tumors with a rich vascular supply than their normal litter mate controls (33). This was a great surprise, particularly

when one considers the large numbers of papers detailing inhibition of vascular development under conditions where the function of $\alpha v\beta 3$ integrin is perturbed by specific antagonists (36–38). Our data seem to reconcile these contradictory results. We show this in model form (Fig. 5). In our model, the $\alpha v\beta 3$, $\alpha 3\beta 1$, and $\alpha 6\beta 1$ integrins are ''activated'' by interaction with $\alpha 4$ laminin ligand. A downstream consequence of such interaction is angiogenesis. Furthermore, as we have discussed above, under normal conditions, $\alpha v\beta 3$ integrin modulates the function of $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins and vice versa. In the model, the antibody LM609 inhibits the function of the $\alpha v\beta 3$ integrin and, in turn, indirectly perturbs activity of $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins. In the complete absence of $\alpha v\beta 3$ integrin, the $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins trigger pathways that are necessary for angiogenesis. Moreover, one could also envisage that in the absence of $\alpha v\beta 3$ integrin, the activity of the $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins may even be enhanced, leading to the observed increase in tumorigenesis in knockout animals (33).

In summary, we have characterized an intricate crosstalk among the integrin heterodimers involved in endothelial cell–$\alpha 4$ laminin interaction. It is now our goal to assess whether function-blocking antibodies against the $\alpha 4$ laminin subunit, such as antibody 2A3, or peptides that compete with endogenous $\alpha 4$ subunit containing laminin heterotrimers for integrin binding, may have therapeutic use as angiogenesis inhibitors.

We thank Dr. Michael Pins for the tumor tissue used in our analyses. This work was supported by National Institutes of Health Grants RO1 HL67016 and PO1 DE12328 and American Heart Association Grants 0151136Z (to J.C.R.J.) and PO1 AR44012 (to G.S.H.). A.M.G. was supported by a Minority Postdoctoral Supplement to PO1 DE12328.

1. Aumailley, M. & Smyth, N. (1998) *J. Anat.* **193,** 1–21.
2. Timpl, R. & Brown, J. C. (1994) *Matrix Biol.* **14,** 275–281.
3. Tunggal, P., Smyth, N., Paulsson, M. & Ott, M.-C. (2000) *Microsc. Res. Tech.* **51,** 214–227.
4. Smirnov, S., McDearmon, E., Shaohua, L., Ervasti, J., Tryggvason, K. & Yurchenco, P. D. (2002) *J. Biol. Chem.* **277,** 18928–18937.
5. Patton, B. L. (2000) *Microsc. Res. Tech.* **51,** 247–261.
6. Baker, S. E., Hopkinson, S. B., Fitchmun, M., Andreason, G. L., Frasier, F., Plopper, G., Quaranta, V. & Jones, J. C. R. (1996) *J. Cell Sci.* **109,** 2509–2520.
7. McGowan, K. & Marinkovich, M. P. (2000) *Microsc. Res. Tech.* **51,** 262–279.
8. Niimi, T., Kumagai, C., Okano, M. & Kitagawa, Y. (1997) *Matrix Biol.* **16,** 223–230.
9. Richards, A., Al-Imara, L. & Pope, F. M. (1996) *Eur. J. Biochem.* **238,** 813–821.
10. Frieser, M., Nöckel, H., Pausch, F., Röder, C., Hahn, A., Deutzmann, R. & Sorokin, L. M. (1997) *Eur. J Biochem.* **246,** 727–735.
11. Talts, J. F., Sasaki, T., Miosge, N., Gohring, W., Mann, K., Mayne, R. & Timpl, R. (2000) *J. Biol. Chem.* **275,** 35192–35199.
12. Iivanainen, A., Sainio, H. & Tryggvason, K. (1995) *FEBS Lett.* **365,** 183–188.
13. Miner, J. H., Patton, B. L., Lentz, S. I., Gilbert, D. J., Snider, W. D., Jenkins, N. A., Copeland, N. G. & Sanes, J. R. (1997) *J. Cell Biol.* **137,** 685–701.
14. Liu, J. & Mayne, R. (1996) *Matrix Biol.* **15,** 433–437.
15. Iivanainen, A., Kortesmaa, J., Sahlberg, C., Morita, T., Bergmann, U., Thesleff, I. & Tryggvason, K. (1997) *J. Biol. Chem.* **272,** 27862–27868.
16. Tokida, Y., Aratani, Y., Morita, A. & Kitagawa, Y. (1990) *J. Biol. Chem.* **265,** 18123–18129.
17. Fujiwara, H., Kikkawa, Y., Sanzen, N. & Sekiguchi, K. (2001) *J. Biol. Chem.* **276,** 17550–17558.
18. Gonzales, M., Weksler, B., Tsuruta, D., Golman, R. D., Yoon, K. J., Hopkinson, S. B., Flitney, F. W. & Jones, J. C. R. (2001) *Mol. Biol. Cell* **12,** 85–100.
19. Schweitzer, K. M., Vicart, P., Delouis, C., Paulin, D., Drager, D., Langenhuijsen, M. M. & Weksler, B. (1997) *Lab. Invest.* **76,** 25–36.
20. Baker, S. E., DiPasquale, A., Stock, E. L., Plopper, G., Quaranta, V., Fitchmun, M. & Jones, J. C. R. (1996) *Exp. Cell Res.* **228,** 262–270.
21. Laemmli, U. K. (1970) *Nature* **277,** 680–685.
22. Harlow, E. & Lane, D. (1988) in *Antibodies: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 92–121.
23. Klatte, D. H., Kurpakus, M. A., Grelling, K. A. & Jones, J. C. R. (1989) *J. Cell Biol.* **109,** 3377–3390.
24. Yang, J., Nagavarapu, U., Relloma, K., Sjaastad, M. D., Moss, W. C., Passaniti, A. & Herron, G. S. (2001) *Nat. Biotech.* **19,** 219–224.
25. Stromblad, S. & Cheresh, D. A. (1996) *Trends Cell Biol.* **6,** 462–468.
26. Lyden, D., Hattori, K., Dias, S., Costa, C., Blaikie, P., Butros, L., Chadburn, A., Heissig, B., Marks, W., Witte, L., *et al.* (2001) *Nat. Med.* **7,** 1194–1201.
27. Blystone, S. D., Graham, I. L., Lindberg, F. P. & Brown, E. J. (1994) *J. Cell Biol.* **127,** 1129–1137.
28. Hodivala-Dilke, K. M., DiPersio, C. M., Kreidberg, J. A. & Hynes, R. O. (1998) *J. Cell Biol.* **142,** 1357–1369.
29. Eliceiri, B. P. & Cheresh, D. A. (1999) *J. Clin. Invest.* **103,** 1227–1230.
30. Thyboll, J., Kortesmaa, J., Cao, R., Soininen, R., Wang, L., Iivanainen, A., Sorokin, L., Rinsling, M., Cao, Y. & Tryggvason, K. (2002) *Mol. Cell. Biol.* **22,** 1194–1202.
31. Brooks, P. C., Clark, R. A. & Cheresh, D. A. (1994) *Science* **264,** 569–571.
32. Ruoslahti, E. & Engvall, E. (1997) *J. Clin. Invest.* **100,** S53–S56.
33. Reynolds, L. E., Wyder, L., Lively, J. C., Taverna, D., Robinson, S. D., Huang, X., Sheppard, D., Hynes, R. O. & Hodivala-Dilke, K. M. (2002) *Nat. Med.* **8,** 27–34.
34. Bader, B. L., Rayburn, H., Crowley, D. & Hynes, R. O. (1998) *Cell* **95,** 507–519.
35. Hodivala-Dilke, K. M., McHugh, K. P., Tsakiris, D. A., Rayburn, H., Crowley, D., Ullman-Cullere, M., Ross, P. F., Coller, B. S., Teitelbaum, S. & Hynes, R. O. (1999) *J. Clin. Invest.* **103,** 229–238.
36. Brooks, P. C., Montgomery, A. M. P., Rosenfeld, M., Reisfeld, R. A., Hu, T., Klier, G. & Cheresh, D. A. (1994) *Cell* **79,** 1157–1164.
37. Drake, C. J., Cheresh, D. A. & Little, C. D. (1995) *J. Cell Sci.* **108,** 2655–2661.
38. Brooks, P. C., Stromblad, S., Klemke, R., Visscher, D., Sarkar, F. H. & Cheresh, D. A. (1995) *J. Clin. Invest.* **96,** 1815–1822.