The Lymphangiogenic Vascular Endothelial Growth Factors VEGF-C and -D Are Ligands for the Integrin α9β1*

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Mice homozygous for a null mutation of the integrin α9β1 subunit die 6–12 days after birth from bilateral chylothoraces suggesting an underlying defect in lymphatic development. However, until now the mechanisms by which the integrin α9β1 modulates lymphangiogenesis have not been described. In this study we show that adhesion to and migration on the lymphangiogenic vascular endothelial growth factors (VEGF-C and -D) are α9β1-dependent. Mouse embryonic fibroblasts and human colon carcinoma cells (SW-480) transfected to express α9β1 adhered and/or migrated on both growth factors in a concentration-dependent fashion, and both adhesion and migration were abrogated by anti-α9β1 function-blocking antibody. In SW-480 cells, which lack cognate receptors for VEGF-C and -D, both growth factors induced α9β1-dependent Erk and paxillin phosphorylation. Human microvascular endothelial cells, which express both α9β1 and VEGF-R3, also adhered to and migrated on both growth factors, and both responses were blocked by anti-α9β1 antibody. Furthermore, in a solid phase binding assay recombinant VEGF-C and -D bound to purified α9β1 integrin in a dose- and cation-dependent fashion showing that VEGF-C and VEGF-D are ligands for the integrin α9β1. The interaction between α9β1 and VEGF-C and/or -D may begin to explain the abnormal lymphatic phenotype of the α9 knock-out mice.

Integrins are heterodimeric transmembrane proteins, which serve as receptors for a variety of spatially fixed extracellular ligands (1). By virtue of their dual roles in adhesion and signaling and because of their close association with the actin cytoskeleton, integrins play important roles in regulating cell shape and cell migration (2, 3). In vertebrates there are 8 identified integrin β subunits and 18 α subunits that form at least 25 different heterodimers (4). The integrin α9 subunit forms a single heterodimer with β1 and is expressed in epithelial cells, smooth and skeletal muscle, neutrophils, and a subset of endothelial cells (5, 6). In vitro, the principal demonstrated function of α9β1 is acceleration of cell migration, an effect that depends on unique sequences within the α9 cytoplasmic domain (7, 8).

In a previous study we inactivated the α9 subunit in mice to better understand the function of α9β1. In these mice lymph leaked into the pleural space (chylothorax), and the mice died 6–12 days after birth. This phenotype was an unexpected finding which indicated that lymph vessel development and/or function was abnormal (9). On gross inspection, the thoracic duct and peripheral lymphatic vessels were present, but their integrity was compromised, as evidenced by chylothoraces and edema of the thoracic dermis, skeletal muscle, and pleural surface. To date the molecular mechanisms underlying the role of α9β1 in lymphatic development and/or function remain unexplained.

The vascular endothelial growth factors (VEGF-C and -D)1 are important mediators of lymphatic development (10, 11). VEGF-C and -D constitute a subfamily of VEGF proteins characterized by 48% overall homology, receptor specificity (VEGF-R3), and highly homologous cysteine-rich C-terminal regions (11, 12). These growth factors are secreted as pro-proteins and after enzymatic cleavage to their mature form (13–15), signal through VEGF receptors 2 (VEGF-R2) and 3 (VEGF-R3), inducing angiogenesis and lymphangiogenesis, respectively (16–22). The importance of these VEGF proteins in lymphangiogenesis was demonstrated by their transgenic overexpression in skin, resulting in dermal lymphatic hyperplasia, which could be blocked by soluble VEGF-R3-Ig (23).

Therefore, in this study, we hypothesized that the lymphatic abnormality in α9 knock-out mice could be explained by an interaction between α9β1 and VEGF-C and/or -D. To address this question we used α9-transfected cell lines and primary microvascular endothelial cells to assess in vitro cell adhesion, migration, and receptor signaling and purified α9β1 and VEGF-C or -D protein for solid phase binding assays. We found that α9-transfected cells and primary microvascular endothelial cells, which endogenously express α9β1, utilize α9β1 to adhere to and migrate on VEGF-C and -D. This effect was inhibited by the specific α9β1-blocking antibody Y9A2 and siRNA silencing of α9 protein expression. Furthermore, VEGF-C and -D directly bound to α9β1 in a solid phase protein binding assay and activated α9β1 signaling, as evidenced by Erk 1/2 and paxillin phosphorylation that was inhibited by anti-α9β1 antibody. These novel findings therefore identify the growth factors VEGF-C and -D as ligands for α9β1 and provide a potential explanation for the abnormal lymphatic phenotype of the α9 knock-out mouse.

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‡ The abbreviations used are: VEGF, vascular endothelial growth factor; VEGF-R, vascular endothelial growth factor receptor; MEF, mouse embryonic fibroblasts; HMVEC, human microvascular endothelial cells; BSA, bovine serum albumin; TfnuR3α, α9-specific ligand, recombinant third fibronectin repeat of tenascin C in which RGD is mutated to RAA; siRNA, small interfering RNA; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; NaVO₃, sodium orthovanadate; Erk, extracellular signal-regulated kinase.
EXPERIMENTAL PROCEDURES

Materials—Human VEGF-C and -D were purchased from R&D Systems. Rabbit anti-human antibody to VEGF-C was purchased from IBL (Gunma, Japan). Rabbit polyclonal antibody to VEGF-R3 (M-20) and rabbit anti-human antibody to VEGF-D (sc-13085) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-VEGF-C antibody 9D9f9 was a kind gift from Dr. K. Alitalo (University of Helsinki, Finland). The VEGF-R3-blocking chemical MAZ-51 was purchased from Alexis Biochemicals (San Diego, CA). Rabbit polyclonal anti-paxillin, anti-paxillin pY31, and anti-Erk1/2/pTyr202/403 were purchased from Biosource (Camarillo, CA). Mouse anti-Erk2 was purchased from BD Biosciences and anti-phosphotyrosine antibody (4G10) was purchased from Cell Signaling Technology (Danvers, MA). Anti-dase-conjugated goat anti-rabbit, goat anti-mouse IgG, phycoerythrin-conjugated goat anti-mouse, and biotinylated rat anti-mouse antibodies and streptavidin-horseradish peroxidase were purchased from Jackson Immunoresearch Laboratories (West Grove, PA).

Cells and Cell Culture—9d and mock-transfected mouse embryonic fibroblasts (MEF) and SW-480 cells were made as described previously (8, 24). Cells were grown in Dulbecco’s minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Hyclone), 1% penicillin/streptomycin (Sigma), and stable clones maintained with 2.5 g/ml puromycin (Invitrogen, MEF) or 1 mg/ml G418 (Sigma, SW-480). Primary adult human dermal microvascular endothelial cells (HMVEC, Cambrex, East Rutherford, NJ) were grown in cell-specific growth factor-supplemented nutrient media (Cambrex, EBM-2). Schneider Drosophila S2 cells (Invitrogen) were used for production of recombinant VEGF-C and -D and were grown to 70–80% confluence in full growth media and then treated with 20 mg/ml of cycloheximide. Cells were trypsinized, kept in suspension for 2 h in 6-well plates coated with 1% BSA (Sigma). Subsequently, 1 × 10⁶ cells/ml untreated or pretreated with relevant blocking antibodies were seeded into separate 6-well plates coated with either 1% BSA, VEGF-C or -D (3 μg/ml), or Tfn3R3A (2.5 μg/ml). Tfn3R3A is an anti-VEGF specific ligand, which is a recombinant form of the third fibronectin type 3 repeat of tenasin C in which the arginine-glycine-aspartic acid sequence is mutated to RAA, as described previously (25, 26). After 60 min, cells were washed with PBS/NaV (1 mM) and then lysed with a buffer containing 20 mg/ml Tris, pH 7.4, 150 mm NaCl, 5 mm EDTA, 1% Triton X, 25 mg/ml NaV, 10% glycerol, 1 mm phenylmethylsulfonyl fluoride, 1 mm NaV, and protease inhibitors (Complete Mini EDTA-free, Roche Applied Science). Lysates were centrifuged at 14,000 g at 4 °C for 10 min, and Western blotting was performed as outlined above.

Flow Cytometry—Cultured cells were trypsinized, washed with PBS, blocked with normal goat serum at 4 °C for 10 min, incubated with primary antibody for 20 min at 4 °C and then with phycoerythrin-conjugated goat anti-mouse antibody. Labeled cells were suspended in PBS containing 0.5% w/v sodium EDTA-free Roche Applied Science). Lysates were centrifuged at 14,000 g at 4 °C for 10 min, and Western blotting was performed as outlined above.

Adhesion Assay—96-well non-tissue culture flat-bottomed microtiter plates (ICN, Linbro/Titertek, Aurora, OH) were seeded overnight with monolayer cultures of S2 cells, grown in 90% Schneider Drosophila S2 medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Sigma), and stable clones maintained with 2.5 g/ml puromycin (Invitrogen, MEF) or 1 μg/ml G418 (Sigma, SW-480). Cells were grown to 70–80% confluence in full growth media and then treated with 20 mg/ml of cycloheximide. Cells were trypsinized, kept in suspension for 2 h in 6-well plates coated with 1% BSA (Sigma). Subsequently, 1 × 10⁶ cells/ml untreated or pretreated with relevant blocking antibodies were seeded into separate 6-well plates coated with either 1% BSA, VEGF-C or -D (3 μg/ml), or Tfn3R3A (2.5 μg/ml). Tfn3R3A is an anti-VEGF specific ligand, which is a recombinant form of the third fibronectin type 3 repeat of tenasin C in which the arginine-glycine-aspartic acid sequence is mutated to RAA, as described previously (25, 26). After 60 min, cells were washed with PBS/NaV (1 mM) and then lysed with a buffer containing 20 mg/ml Tris, pH 7.4, 150 mm NaCl, 5 mm EDTA, 1% Triton X, 25 mg/ml NaV, 10% glycerol, 1 mm phenylmethylsulfonyl fluoride, 1 mm NaV, and protease inhibitors (Complete Mini EDTA-free, Roche Applied Science). Lysates were centrifuged at 14,000 g at 4 °C for 10 min, and Western blotting was performed as outlined above.

Migration Assay—The undersurfaces of 12-well, 8 mm Transwell plates (Corning, Costar, Cambridge, MA) were coated with the relevant VEGF-C or VEGF-D as substrate were performed using transfected cells in which aβ1 was successfully silenced and compared with untransfected and transfected cells in which aβ1 was not silenced.

Production of Non-blocking Monoclonal Antibody to aβ1—A non-
methionine were then precleared with protein A-Sepharose for 1 h. Infected SW-480 cells (24) were lysed with buffer containing 100 mM NaCl, 300 mM Na2PO4, pH 8 (29). Following five washes with PBS/1% BSA/0.05% Tween, the proteins were separated by 15% SDS-PAGE under reducing conditions, and Western blotted with an anti-phosphotyrosine antibody. IP, immunoprecipitate. Molecular mass markers (kDa) are indicated to the left of all panels.

**Production and Purification of VEGF-C and -D Proteins.** VEGF-C and -D were purified from supernatant of transfected *Drosophila* S2 cells by Ni²⁺ affinity chromatography. Proteins were separated by 15% SDS-PAGE under reducing conditions and analyzed by Western blot analysis (A) with V5 antibody (left panels) and VEGF-C and -D-specific antibodies (right panels) and silver staining (B). C, phosphoryrosine blot from control HMVEC and HMVEC incubated with V5-tagged VEGF-C or -D. Lysates were immunoprecipitated with either no antibody or antibody against VEGF-R3, separated by 8% SDS-PAGE under reducing conditions, and Western blotted with an anti-phosphotyrosine antibody. IP, immunoprecipitate.

**Results**

**Production and Purification of VEGF-C and -D**—Fully processed VEGF-C and -D proteins were purified from transfected *Drosophila* S2 cells. Western blots (Fig. 1A) of secreted and purified VEGF-C (top panel) and VEGF-D (bottom panel) with anti-V5 antibody (left panels) demonstrate proteins with the expected molecular mass for VEGF-C (or -D) tagged with V5 and His (~23–24 kDa). In addition, immunoblotting with VEGF-C- and -D-specific antibodies (right panels) showed that the V5-tagged proteins were indeed VEGF-C and -D. The purity of each recombinant protein was determined by silver staining of 15% SDS-polyacrylamide gels as shown in Fig. 1B. VEGF-C and -D-5 induced phosphorylation of their cognate receptor, VEGF-R3. Fig. 1C shows the expected VEGF-R3 isoforms and confirms the activity of the purified VEGF proteins.

**Cell Adhesion to VEGF-C and -D is α9β1-Dependent**—Adhesion assays were performed using α9- and mock-transfected MEF and SW-480 cells that do not express VEGF-R2 or -R3, (11, 30) (data not shown). Flow cytometry with the anti-α9β1 antibody Y9A2 showed robust expression of α9β1 in both α9-transfected cell types and no expression in mock transfectants (Fig. 2A). α9-Transfected MEF demonstrated a concentration-dependent adhesion to both VEGF-C and -D (Fig. 2, B and C). This effect was abolished by the α9β1-blocking antibody Y9A2 (p < 0.05). In contrast, mock-transfected MEF did not adhere to either VEGF protein above background levels of attachment to BSA-coated wells. Similarly, α9β1-dependent adhesion on both VEGF-D (Fig. 2D) and VEGF-C (data not shown) was demonstrated in SW-480 cells.

**Binding of VEGF-C or -D Activates α9β1 Integrin**—We have shown previously that in SW-480 cells, activation of the α9β1
integrin by established ligands results in phosphorylation of Erk 1/2 and paxillin. To assess whether binding of VEGF-C or -D similarly results in $\alpha_9\beta_1$-induced signaling, phosphorylation of these proteins was compared in mock- and $\alpha_9\beta_1$-transfected SW-480 cells in the presence or absence of the $\alpha_9\beta_1$-blocking antibody. Fig. 3A shows that after 15 min of $\alpha_9\beta_1$ transfectants binding to adherent Tnfn3RAA (lanes 3 and 4), VEGF-C (lanes 5 and 6) or VEGF-D (lanes 7 and 8), Erk 1/2 phosphorylation was increased in an $\alpha_9\beta_1$-dependent fashion. Similar to 1% BSA (lane 1), this response to VEGF was not seen in mock-transfected cells (lane 2). Paxillin phosphorylation was also induced after 15 min in $\alpha_9\beta_1$ transfectants and was inhibited by the $\alpha_9\beta_1$-blocking antibody (Fig. 3B).

$\alpha_9\beta_1$-Dependent Cell Adhesion Is Demonstrable in Primary Endothelial Cells—To verify the biological significance of the results obtained with the transfected cell lines and to understand the role of $\alpha_9\beta_1$ in adhesion to VEGF-C and -D in the presence of their cognate receptor VEGF-R3, HMVEC were also studied. HMVEC were found to express both VEGF-R3 and $\alpha_9\beta_1$ as measured by flow cytometry (Fig. 4A and B). Essentially, all of the cells expressed VEGF-R3 (Fig. 4A), belying their lymphatic origin, whereas the expression of $\alpha_9\beta_1$ varied from 40–55% (Fig. 4B) depending on the lot purchased and decreased with increasing passage number. As a result, all experiments were performed between passages 3 and 7. Like $\alpha_9\beta_1$-transfected cell lines, HMVEC demonstrated concentration-dependent adhesion to both VEGF-C (Fig. 4C) and VEGF-D (Fig. 4D). Adhesion to both substrates was inhibited by the $\alpha_9\beta_1$-blocking antibody Y9A2 ($p < 0.05$) to the levels measured in wells coated with 1% BSA, demonstrating that the presence of VEGF-R3 is not sufficient to mediate cell adhesion to VEGF-C or -D.

Similar results were obtained when $\alpha_9\beta_1$ was silenced using siRNA transfection (Fig. 5). The effectiveness of siRNA directed against exon 4 of $\alpha_9$ ($\alpha_9$ siRNA) was assessed by measuring $\alpha_9$ expression on the cell surface by flow cytometry (Fig. 5A). The expression of $\alpha_9$ in these cells was compared against cells transfected with mock siRNA and cells not treated with siRNA (No siRNA). Adhesion assays were then performed using these cell populations. Fig. 5B shows that knock-down of $\alpha_9$ in HMVEC cells significantly inhibits cell adhesion on both VEGF-C and VEGF-D. Mock siRNA cells adhered to VEGF-C or VEGF-D substrate to the same degree as non-transfected HMVEC.

Cell Migration on VEGF-C and -D Is also $\alpha_9\beta_1$-Dependent—We have shown previously that one of the principal func-
VEGF-C and -D Are Ligands for Integrin α9β1

Production of Non-blocking α9β1 Antibody—A mouse anti-human monoclonal antibody (A9A1) was produced to serve as a detection antibody for α9β1 in VEGF-C or -D solid phase binding assays. Flow cytometry using the antibody A9A1 shows detection of the α9β1 integrin on α9-transfected MEF to a similar level as that of Y9A2, an established α9β1 integrin antibody (Fig. 7A). To further characterize its specificity, A9A1 was used to immunoprecipitate lysates of [35S]methionine-labeled α9-transfected SW-480 cells. Fig. 7B shows an autoradiograph of immunoprecipitated protein samples separated by 8% SDS-PAGE where the α9 and β1 bands of the heterodimer are clearly seen. Fig. 7C shows that in contrast to Y9A2, a blocking antibody to α9β1, A9A1 did not inhibit adhesion of α9-transfected MEF to the α9β1-specific ligand Tfn3RAA. This antibody was thus suitable for use in α9β1-VEGF binding assays.

Purification of Active Human α9β1 Integrin—To determine whether α9β1 directly binds to VEGF-C and/or -D, solid phase binding assays were performed using purified VEGF-C or -D and α9β1 purified from α9-transfected SW-480 cells (24) using an A9A1 affinity column. Fig. 8, A and B, shows a Coomassie stain (reduced, 8% SDS-PAGE) and immunoblot of eluted α9β1 (α9 ~160 kDa, β1 ~130 kDa). This purified integrin was still capable of binding to the α9β1-specific ligand Tfn3RAA, an effect that was inhibited by 10 mM EDTA (Fig. 8C).

VEGF-C and -D Bind the Integrin α9β1—Solid phase binding assays demonstrated robust concentration-dependent adhesion of α9β1 to VEGF-C and -D, both at 5 μg/ml (Fig. 9, A and B). In both cases, binding was completely inhibited by chelating divalent cations (EDTA, 10 mM) as expected for authentic integrin-ligand interactions. The irrelevant integrin αβ6 (28) showed no binding to either VEGF-C or -D.

DISCUSSION

In this study we found that α9-transfected cells (lacking VEGF receptors) adhere and migrate on fully processed VEGF-C and -D. These cellular responses require functional α9β1 because they are inhibited by a blocking antibody. These results alone suggest that an interaction between α9β1 and the VEGF-C and -D proteins can affect cell behavior in the absence of VEGF-R3, the cognate receptor of the growth factors. Importantly, α9β1 was also shown to mediate adhesion and migration on VEGF-C and -D in primary human endothelial cells that co-express α9β1 and VEGF-R3. Inhibition of migration by the α9β1 antibody and/or by blocking VEGF-R3 suggests that VEGF-R3 is not sufficient to mediate maximal migration on VEGF-C or -D. Solid phase binding assays of purified VEGF-C or -D and α9β1 verified that these cell functions are a result of VEGF-C and -D binding α9β1 in an integrin-specific and cation-dependent manner. Moreover, binding of VEGF-C or -D to α9β1 leads to induction of downstream signals as demonstrated by α9β1-dependent Erk 1/2 and paxillin phosphorylation. The finding that growth factors (VEGF-C and -D) bind to an integrin (α9β1) highlights a novel and important mechanism of integrin-growth factor interaction.

A number of previous studies have described cooperative interactions between integrins and growth factors (reviewed in Refs. 31 and 32). Growth factor ligation of its cognate receptor has been shown to lead to close physical association with integrins. For example, ligation of the platelet-derived growth factor receptor not only allowed its co-immunoprecipitation with the αvβ3 integrin but also potentiated αvβ3-dependent cell migration (33). In addition, input from VEGF-R2, stimulated by VEGF-A, has been shown to activate the αv integrins αvβ3 and αvβ5 and the β1 integrins α5β1 and α2β1 (34).

Activated integrins have also been shown to modulate growth factor protein and receptor expression and signaling. For example, integrin αvβ4 increases VEGF-A translation in breast carcinoma cells through inactivation of the translational repressor 4E-BP1 (35). In addition, integrin-matrix interactions modulate expression of VEGF and fibroblast growth factor receptor 1 and 2 expression on HMVEC (36). The functional importance of growth factor/integrin interactions has also been demonstrated in vivo where blocking antibodies to αvβ3 and αvβ5 integrins inhibit basic fibroblast growth factor- and VEGF-induced angiogenesis, respectively (37). Finally, mice lacking the integrin αvβ3 are specifically protected from the increases in vascular permeability induced by VEGF-A, again presumably through cross-talk with VEGF-R2 (38).

In the current study, we describe a novel mechanism of growth factor-integrin interaction, direct ligation of an integrin by a growth factor. This interaction thus provides a mechanism for VEGF-C and -D to affect directly cell behavior (i.e. cell adhesion and migration) even in the absence of their cognate receptor, VEGF-R3. Furthermore, even in cells expressing the

FIG. 3. Binding of VEGF-C and -D to α9β1 activates the integrin. Mock- or α9-transfected SW-480 cells were plated on various proteins for 15 min, and cell lysates were obtained. Following BCA protein quantification equal amounts of protein were separated under reducing conditions on 10% SDS-PAGE. A, SW-480 cells (upper panel) were plated for 15 min on 1% BSA, the α9-specific ligand Tfn3RAA (RAA), VEGF-D, or VEGF-C in the presence or absence of the α9-blocking antibody (anti-α9 Ab, 20 μg/ml) and immunoblotted with phosho-Erk 1/2 (pErk 1/2) (upper panel). The polyvinylidene difluoride membrane was re-touched with total Erk 2 to ensure equal protein loading (lower panel). B, SW-480 cells were plated for 15 min on VEGF-D or VEGF-C in the presence or absence of the α9-blocking antibody (20 μg/ml) and immunoblotted with phospho-paxillin (pPaxillin) (upper panel). The polyvinylidene difluoride membrane was re-touched with total paxillin to ensure equal protein loading (lower panel).
cognate receptor, ligation of the integrin αβ1 is required for stable cell adhesion to these growth factors and substantially enhances cell migration across them. Our results do not exclude the possibility that αβ1 may modulate cell function by activating another receptor capable of binding VEGF or inhibiting a VEGF-R3 suppressor. However, taken together these results strongly suggest that VEGF-C and -D directly bind to αβ1. We speculate that simultaneous binding of both αβ1 and VEGF-R3 by VEGF-C and/or -D, which is present at high concentration in the extracellular matrix, results in co-clustering of both receptors, facilitating cooperative interactions between the two receptors as demonstrated by our cell migration data. The specific binding sites on VEGF-C and -D and their relationship to the binding sites on other αβ1 ligands, such as tenascin-C and osteopontin, are as yet undetermined. Because of the lack of a conserved binding sequence for known αβ1 ligands, determination of this site for VEGF-C or -D would be difficult. Because the VEGF family members share a VEGF homology domain our results suggest that other VEGF family member proteins may also interact with αβ1. It is also clear that in addition to integrins, other cell surface proteins can modulate VEGF responses, such as the other VEGF receptors (39) and the neuropilins (40). The potential interactions between these modulating proteins and αβ1 remain to be determined.

Normal vessel development requires the coordinated function of both cellular and extracellular regulatory and effector proteins to ensure an optimal milieu for correct vessel morphogenesis and function (41). The molecular regulators of angiogenesis include the VEGF family of proteins and receptors...
VEGF-C and -D Are Ligands for Integrin α9β1

Fig. 6. Cell migration on VEGF-C and -D is α9β1-dependent. Migration assays on VEGF-C or -D, coated on the lower surface of a Transwell at various concentrations, were performed with 1% fetal calf serum as a chemotactic factor. VEGF-C (A) or VEGF-D (B) was used as substrate for migration assays with mock-transfected (diagonal bars) or α9-transfected (dark bars) MEF in the absence or presence (stippled bars) of Y9A2. Similar assays were performed in HMVEC on VEGF-C (C) or VEGF-D (D) substrate in the absence (dark bars) or presence (stippled bars) of the α9β1-blocking antibody. E, VEGF-D was used as substrate for migration assays with HMVEC in the absence (dark bars) or presence (shaded bars) of the α9β1-blocking antibody Y9A2 and/or the VEGF-3-blocking drug MAZ-51. *, p < 0.05 compared with cells treated with Y9A2; #, p < 0.05 compared with cells treated with MAZ-51 alone. Ab, antibody; hpF, high power fields.

Fig. 7. A9A1, a specific non-blocking antibody to α9β1. A, flow cytometry analysis of α9-transfected (line) and mock-transfected (shaded area) MEF using the α9 antibody A9A1 (right) compared with the α9β1-specific antibody Y9A2 (left). B, autoradiograph of 35S-labeled α9-transfected SW-480 cell lysates immunoprecipitated with 20 μg of A9A1. The observed bands represent the α9 and β1 subunits of the integrin. C, Tnfn3RAA, an α9-specific ligand, was used as substrate for adhesion assays in α9-transfected MEF in the absence (dark bars) or presence (diagonal bars) of the A9A1 antibody and compared with the blocking antibody Y9A2 (stippled bars). *, p < 0.05 compared with cells treated with Y9A2. Ab, antibody.

VEGF-R1, -R2, -R3), acidic and basic fibroblast growth factors, angiopoietins and Tie receptors, ephrins, integrins, and matrix metalloproteases (10, 42). The molecules that regulate lymphangiogenesis have not been as well characterized. However, it is clear that VEGF-C and -D are key modulators of lymphatic development and function (21, 22), although their relative roles remain to be determined (19). These growth factors induce lymphatic endothelial cell growth, survival, and migration in vitro (16) and lymphangiogenesis in vivo (43), mediated at least in part through their activation of VEGF-R3. In addition, loss of function mutations in VEGF-R3 have been described in a significant subgroup of patients with congenital human lymphedema (44).

The α9β1 integrin has now been described to interact with a relatively large number of ligands including tenascin C, osteopontin, vascular cell adhesion molecule-1, coagulation factor XIII, and several members of the ADAMs family of transmembrane metalloproteinas (7, 24, 45–47). The biological significance of α9β1 interactions with most of these ligands remains to be determined. In contrast, we now show that the ligands VEGF-C and -D play a role in endothelial cell adhesion and migration, key cellular functions required for lymphangiogen-
FIG. 8. Purification of active α9β1.

α9β1 integrin was purified from cell lysates of α9-transfected SW-480 cells by affinity chromatography using an α9β1 antibody (A9A1) column. Proteins under reducing conditions were separated by 8% SDS-PAGE and analyzed by silver staining (A) and Western blot analysis with 1057, rabbit polyclonal antibody to α9β1 (B). Molecular mass markers (kDa) are indicated. C, Tfn3n3RAA, an α9-specific ligand, was used as substrate for binding assays with purified α9β1 in the absence (diamonds) or presence (squares) of 10 mM EDTA.

FIG. 9. VEGF-C and -D bind directly to the integrin α9β1. Purified VEGF-C (A) or VEGF-D (B) was used for solid phase binding assays with purified α9β1 at various concentrations in the absence (diamonds) or presence (squares) of 10 mM EDTA. Similar assays were performed using purified αvβ8, an irrelevant integrin (triangles).

esis. The most dramatic phenotypic feature of α9 null mice is the presence of bilateral chylothoraces, suggesting a functional defect in the collecting lymphatics of the thorax. Subtle edema and the accumulation of lymphocytes around some peripheral lymphatics provide further support for a role of this integrin in lymphangiogenesis (9). The phenotype of VEGF-D null mice is yet to be published; however, it is clear from E10.5 VEGF-C heterozygote mice that VEGF-C is expressed in mesenchymal cells surrounding the jugular vein toward which endothelial cells that already have a lymphatic differentiation (Prox-1-positive) must migrate (48). Given the specialized role that α9β1 plays in accelerated cell migration (8), we speculate that abnormal migration on VEGF-C and/or -D might be responsible for the functional defects that occur in α9 null mice. However, the fact that lymphatic vessels are formed in these mice suggests that either the presence of VEGF-R3 or some other unidentified receptor(s) can partially compensate for the loss of α9β1.

In summary, the findings of this study describe a novel integrin-growth factor interaction whereby the lymphangiogenic proteins VEGF-C and -D bind the integrin α9β1. Although the precise mechanisms by which binding of VEGF-C and/or -D to α9β1 contributes to lymphatic development remain to be fully elucidated, these data strongly support a role for this interaction in explaining the lymphatic defects in α9 knock-out mice.

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REFERENCES
1. Hyne, R. O. (1993) Cell 69, 11–25
2. Friedl, P. (2004) Curr. Opin. Cell Biol. 16, 14–23
3. Ridley, A. J., Schwartz, M. A., Burridge, K., Firtel, R. A., Ginsberg, M. H., Borisy, G., Parsons, J. T., and Horwitz, A. R. (2003) Science 302, 1704–1709
4. Hyne, R. O. (2002) Cell 110, 673–687
5. Palmer, E. L., Ruegg, C., Ferrando, R., Pytela, R., and Sheppard, D. (1993) J. Cell Biol. 123, 1289–1299
6. Tarui, T., Miles, L. A., and Takada, Y. (2001) J. Biol. Chem. 276, 39562–39568
7. Taoka, Y., Chen, J., Yednock, T., and Sheppard, D. (1999) J. Cell Biol. 145, 413–420
8. Young, B. A., Taoka, Y., Liu, S., Askina, K. J., Yokosaki, Y., Thomas, S. M., and Sheppard, D. (2001) Mol. Biol. Cell 12, 3214–3225
9. Huang, X. Z., Wu, J. F., Ferrando, R., Lee, J. H., Wang, Y. L., Farese, R. V., Jr., and Sheppard, D. (2000) Mol. Cell. Biol. 20, 5208–5215
10. Alitalo, K., and Carmeliet, P. (2002) Cancer Cell 1, 219–227
11. Jussila, L., and Alitalo, K. (2002) Physiol. Rev. 82, 673–700
12. Ferrara, N., Gerber, H. P., and LeCouter, J. (2003) Nat. Med. 9, 669–676
13. Achen, M. G., Jeltsch, M., Rak, E., Makinen, T., Vitali, A., Wilks, A. F., Alitalo, K., and Stacke, S. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 548–553
14. Joukov, V., Sorsa, T., Kumar, V., Jeltsch, M., Claesson-Welsh, L., Cao, Y., Saksela, O., Kalkkinen, N., and Alitalo, K. (1997) EMBO J. 16, 3898–3911
15. McColl, B. K., Baldwin, M. E., Roufail, S., Freeman, C., Moritz, R. L., Simpson, R. J., Alitalo, K., Stacke, S. A., and Achen, M. G. (2003) J. Exp. Med. 198, 863–868
16. Makinen, T., Veikkola, T., Mustjoki, S., Karpanen, T., Catiemel, B., Wise, N., Mercer, A., Kowalski, H., Kerjaschki, D., Stacke, S. A., Achen, M. G., and Alitalo, K. (2001) EMBO J. 20, 4762–4773
17. Mandriota, S. J., Jussila, L., Jeltsch, M., Compagni, A., Baetens, D., Prevo, R., Banerji, S., Huarte, J., Montesano, R., Jackson, D. G., Orei, L., Alitalo, K., Christofori, G., and Pepper, M. S. (2001) EMBO J. 20, 672–682
18. Marconneti, L., Marchio, S., Morbidelli, L., Cartecoi, E., Albin, A., Ziche, M., Russolino, P., and Oliviero, S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9671–9676
19. Rissanen, T., Markkanen, J. E., Gruchala, M., Heikura, T., Puranen, A., Kettunen, M. I., Khlova, I., Kauppinen, R. A., Achen, M. G., Stacke, S. A., Alitalo, K., and Yla-Herttuala, S. (2003) Circ. Res. 92, 1098–1106
20. Skobe, M., Hamberg, I. M., Hawighorst, T., Schirmer, M., Wolf, G. L., Alitalo, K., and Detmar, M. (2001) J. Exp. Med. 193, 895–903
21. Stacke, S. A., Caesar, C., Baldwin, M. E., Thornton, G. E., Williams, R. A., Prevo, R., Jackson, D. G., Nishikawa, S., Kubo, H., and Achen, M. G. (2001) Nat. Med. 7, 186–191
22. Suzd, A., Skobe, M., Karkkainen, M. J., Shin, W. S., Beynet, D. P., Rockson, N. B., Dakhil, N., Spilman, S., Goris, M. L., Strauss, H. W., Quertermous, T., Alitalo, K., and Rockson, S. G. (2002) FASEB J. 16, 1985–1987
23. Veikkola, T., Jussila, L., Makinen, T., Karpanen, T., Jeltsch, M., Petrova, T. V., Kubo, H., Thurston, G., McDonald, D. M., Achen, M. G., Stacke, S. A., and Alitalo, K. (2001) EMBO J. 20, 1223–1231
24. Yokosaki, Y., Palmer, E. L., Prieto, A. L., Crossin, K. L., Bourdon, M. A., Pytela, R., and Sheppard, D. (1994) J. Biol. Chem. 269, 26691–26696
25. Prieto, A. L., Edelman, G. M., and Crossin, K. L. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10154–10158
26. Yokosaki, Y., Matsuura, N., Higashiyama, S., Murakami, I., Ohara, M., Ya-
makido, M., Shigeto, N., Chen, J., and Sheppard, D. (1998) *J. Biol. Chem.* **273**, 11423–11428.

27. Wang, A., Yokosaki, Y., Ferrando, R., Balmes, J., and Sheppard, D. (1996) *Am. J. Respir. Cell Mol. Biol.* **15**, 664–672.

28. Weinacker, A., Chen, A., Agrez, M., Cone, R. I., Nishimura, S., Wayner, E., Pytel, R., and Sheppard, D. (1994) *J. Biol. Chem.* **269**, 6940–6948.

29. Munger, J. S., Huang, X., Kawakatsu, H., Griffiths, M. J., Dalton, S. L., Wu, J., Pittet, J. F., Kaminiski, N., Garat, C., Matthay, M. A., Rifkin, D. B., and Sheppard, D. (1999) *Cell* **96**, 319–328.

30. Witmer, A. N., Dai, J., Weich, H. A., Vrensen, G. F., and Schlingemann, R. O. (2002) *J. Histochem. Cytochem.* **50**, 767–777.

31. Smyth, S. S., and Patterson, C. (2002) *J. Cell Biol.* **158**, 17–21.

32. Yamada, K. M., and Even-Ram, S. (2002) *Nat. Cell Biol.* **4**, E75–76.

33. Borges, E., Jan, Y., and Ruoslahti, E. (2000) *J. Biol. Chem.* **275**, 39867–39873.

34. Byzova, T. V., Goldman, C. K., Pampori, N., Thomas, K. A., Bett, A., Shattil, S. J., and Plow, E. F. (2000) *Mol. Cell Biol.* **6**, 851–860.

35. Chung, J., Bachelder, R. E., Lipscomb, E. A., Shaw, L. M., and Mercurio, A. M. (1994) *J. Biol. Chem.* **269**, 165–174.

36. Tsou, R., and Isik, F. F. (2001) *Mol. Cell. Biochem.* **224**, 81–89.

37. Brooks, P. C., Montgomery, A. M., Rosenfeld, M., Reisfeld, R. A., Hu, T., Klier, G., and Cheresh, D. A. (1994) *Cell* **79**, 1157–1164.

38. Elceirri, B. P., Puente, X. S., Hood, J. D., Stupack, D. G., Schlaepfer, D. D., Huang, X. Z., Sheppard, D., and Cheresh, D. A. (2002) *J. Cell Biol.* **157**, 149–160.

39. Zeng, H., Dvorak, H. F., and Mukhopadhyay, D. (2001) *J. Biol. Chem.* **276**, 26969–26979.

40. Whittaker, G. R., Limberg, B. J., and Rosenbaum, J. S. (2001) *J. Biol. Chem.* **276**, 25520–25531.

41. Pepper, M. S. (2001) *Arterioscler. Thromb. Vasc. Biol.* **21**, 1104–1117.

42. Yancopoulos, G. D., Davis, S., Gale, N. W., Rudge, J. S., Wiegand, S. J., and Holash, J. (2000) *Nature* **407**, 242–248.

43. Oh, S. J., Jeltsch, M. M., Birkenhager, R., McCarthy, J. E., Weich, H. A., Christ, B., Altunalo, K., and Wiltzing, J. (1997) *Dev. Biol.* **188**, 96–109.

44. Karkkainen, M. J., Ferrell, R. E., Lawrence, E. C., Kinak, M. A., Levinson, K. L., McTigue, M. A., Altunalo, K., and Finegold, D. N. (2000) *Nat. Genet.* **25**, 153–159.

45. Eto, K., Puzon-McLaughlin, W., Sheppard, D., Sehara-Fujisawa, A., Zhang, X. P., and Takada, Y. (2000) *J. Biol. Chem.* **275**, 34922–34930.

46. Majumdar, M., Tarui, T., Shi, B., Akakura, N., Ruf, W., and Takada, Y. (2004) *J. Biol. Chem.* **279**, 37528–37534.

47. Yokosaki, Y., Matsuura, N., Sasaki, T., Murakami, I., Schneider, H., Higashiyama, S., Suitoh, Y., Yamakido, M., Taoaka, Y., and Sheppard, D. (1999) *J. Biol. Chem.* **274**, 36328–36334.

48. Karkkainen, M. J., Haiko, P., Sainio, K., Partanen, J., Taipale, J., Petrova, T. V., Jeltsch, M., Jackson, D. G., Talikka, M., Rauvala, H., Betsholtz, C., and Altunalo, K. (2004) *Nat Immuno* **5**, 74–80.

49. Kirkin, V., Mazitschek, R., Krishnan, J., Steffen, A., Waltenberger, J., Pepper, M. S., Giannis, A., Sleeman, J. P. (2001) *J. Biol. Chem.* **266**, 5530–5540.
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