Dell1 Induces Integrin Signaling and Angiogenesis by Ligation of \(\alpha V\beta 3\)

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Dell1 is a novel extracellular matrix protein encoding three Notch-like epidermal growth factor repeats, an RGD motif, and two discoidin domains. Dell1 is expressed in an endothelial cell-restricted pattern during early development. In studies reported here, recombinant baculovirus Dell1 protein was shown to promote \(\alpha V\beta 3\)-dependent endothelial cell attachment and migration. Attachment of endothelial cells to Dell1 was associated with clustering of \(\alpha V\beta 3\), the formation of focal complexes, and recruitment of talin and vinculin into these complexes. These events were shown to be associated with phosphorylation of proteins in the focal complexes, including the time-dependent phosphorylation of pi25\(^\text{AR}\), MAPK, and Shc. When recombinant Dell1 was evaluated in an in vivo chick chorioallantoic membrane assay, it was found to have potent angiogenic activity. This angiogenic activity was inhibited by a monoclonal antibody directed against \(\alpha V\beta 3\), and an R\-tand mutant Dell1 protein was inactive. Thus Dell1 provides a unique autocrine angiogenic pathway for the embryonic endothelium, and this function is mediated in part by productive ligation of integrin \(\alpha V\beta 3\).

The study of vascular development is important because of the fundamental embryological principles that underlie this complex and essential process, and the potential for therapeutic manipulation of vascular formation in human disease processes. The assembly of endothelial cells into vascular structures requires specific signals from luminal surface receptors that are activated by soluble ligands, and signals from the extracellular matrix mediated through receptors on the abluminal surface of the endothelial cell (1, 2).

Virtually all molecules of the matrix are able to communicate to cells by binding to integrins (3, 4). The integrin receptors are noncovalently associated heterodimeric glycoproteins that reside in the cell membrane where their cytoplasmic domains connect to elements of the cytoskeleton. These molecules support a variety of functions, including cell attachment and migration, and activation of cytoplasmic signaling molecules (4, 5). A number of different integrin receptor complexes have been identified on the surface of endothelial cells, and linked to specific functions (2). Data from recent experiments indicate that interaction of the \(\alpha V\beta 3\) receptor with its ligands is critical for tumor and cytokine-induced angiogenesis, through a cell survival function mediated by this integrin (6). Also, \(\alpha V\beta 3\)-mediated signaling through the Ras-mitogen activated protein kinase (MAPK)\(^3\) pathway, specifically through sustained activation of MAPK, appears to be critical for the angiogenic program (7).

To search for new molecular pathways of vascular development, we have recently characterized a locus identified by an enhancer trap event in a line of transgenic mice (8). The gene located in this locus, Dell1, was shown to be expressed in an endothelial-restricted pattern in early embryonic development. The proteins encoded in the locus were characterized by genomic and cDNA cloning. The isoform encoded by the common transcript, Dell1 major (Del1 MJR), was found to contain a signal sequence, three EGF-like repeats, and two discoidin I-like domains. A less frequent transcript, Dell1 minor (Del1 MNR), was noted to be truncated in the amino-terminal diso- didin I-like domain due to alternative splicing. Both transcripts had an RGD motif in the B loop of the second EGF-like repeat. Preliminary data suggested that Dell1 MJR is a matrix protein, and that the RGD motif could mediate endothelial cell attachment through intigrin ligation. In vitro and in vivo functional studies suggested that Dell1 may have a complex role in the regulation of vascular formation, serving to inhibit endothelial cell differentiation or contribute to the complex process of vascular remodeling. Such a functional role was not easily reconciled with the molecular interaction of Dell1 with the \(\alpha V\beta 3\) integrin receptor.

To further characterize the ability of Dell1 to initiate integrin-mediated events, and to investigate whether Dell1 ligation of integrin receptors triggers signaling pathways similar to other RGD ligands, a series of experiments were conducted with recombinant baculovirus proteins. Cell attachment assays revealed that Dell1 could mediate endothelial cell attachment at concentrations similar to that observed with known matrix proteins such as vitronectin (VN) and fibronectin (FN), and

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1 The abbreviations used are: MAPK, mitogen-activated protein kinase; CAM, chorioallantoic membrane; Dell1 MJR, developmental endothelial locus-1 major isoform; Dell1 MNR, developmental endothelial locus-1 minor isoform; VN, vitronectin; FN, fibronectin; Dell1 RAD, developmental endothelial locus-1 major isoform with RGD→RAD mutation; BAEC, bovine aortic endothelial cells; HUVEC, human umbilical vein endothelial cells; DMEM, Dulbecco's modified Eagle's medium; FAK, focal adhesion kinase; bFGF, basic fibroblast growth factor; DBPS, Dulbecco's phosphate-buffered saline; Shc, C-terminal SH2 domain; ERK, extracellular signal regulated kinases, MEK, MAP or ERK kinase; EGF, epidermal growth factor; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis.
that the majority of this activity was mediated through αvβ3. Also, Del1 was shown to mediate endothelial cell migration through binding to this integrin. Endothelial cells on Del1 were found to form focal complexes that contained phosphorylated proteins characteristic of integrin signaling. In addition, studies employing an in vivo chick CAM assay have documented a novel angiogenic activity of Del1 that is dependent on αvβ3 signaling.

**EXPERIMENTAL PROCEDURES**

**Matrix Components and Chemicals—**Human VN (Life Technologies, Inc., Grand Island, NY) was resuspended in Dulbecco’s phosphate-buffered saline (DPBS) to a final concentration of 14 μg/ml and stored frozen until further use. Cycloheximide, FN, protease inhibitors such as leupeptin, aprotinin, and phenylmethylsulfonyl fluoride, the tyrosine phosphatase inhibitor sodium orthovanadate, and protein A-Sepharose CL-4B were all purchased from Sigma.

**RGD Peptides and Antibodies—**Synthetic peptides were purchased from Life Technologies, Inc. and correspond to sequences GRGDSP, GRGESP, GPGGRGDSPGCA (Pen RGD), where Pen stands for penicillamine. The Del1 peptide represents amino acids 89–106, CEISEAY-RDPTFGYBCK, was synthesized as a linear molecule, and dissolved in dimethyl sulfoxide. Other peptides were dissolved in DPBS at 2 mg/0.1 ml, and stored until further use. Monoclonal antibodies to integrins αvβ3 (clone LM609), αvβ5 (clone PIF6), β1 (clone J1B1a), ε2 (VLA-2), αv (clone CLB-706), α2 (clone JBS2), and α6 (clone CLB-701) were purchased from Chemicon International, Temecula, CA (9–12). A monoclonal antibody to human β3 integrin (GPIIIa) resides 348–426 (clone A5F) was a generous gift from Dr. Peter J. Newman, The Blood Center of Southeastern Wisconsin. Monoclonal antibodies to vinculin (clone VIN-11-5), talin (clone 8d4), and fluorescein isothiocyanate-conjugated phalloidin were purchased from Sigma (13, 14). Monoclonal anti-phosphotyrosine antibody (clone 4G10), anti-Shc polyclonal antibody, and anti-MAPK polyclonal antibody were purchased from Upstate Biotechnology, Lake Placid, NY (15). Phospho-specific MAPK antibody was obtained from New England Biolabs, Beverly, MA. Polyclonal anti-p125FAK (C-20) was purchased from Santa Cruz Biotechnology (16). Cy3-conjugated goat anti-rabbit and goat anti-mouse immunoglobulins were purchased from Jackson Immunoresearch Laboratories, West Grove, PA.

**cDNA Constructs and Recombinant Protein Expression—**Recombinant bacterial Del1 protein was produced in Escherichia coli as described previously (8). Recombinant Del1 protein was expressed in baculovirus. cDNA fragments encoding the Del1 MJR, Del1 MNR, and Del1 MJR protein with RAD substituted for the RGD motif (Del1 RAD) were cloned into the shuttle vector pACG67B6 and transfected into S9 cells with the Baculogold reagents (Pharmingen, San Diego, CA). Del1 RAD was derived by polymerase chain reaction mutagenesis as described previously (17). Recombinant baculovirus expressing Del1 proteins were diluted in appropriate concentrations in DMEM containing 200 μg/ml BSA and placed in the bottom wells of a modified Boyden chemotaxis chamber (Neuro Probe, Cabin John, MD), unless otherwise indicated. Standard assays were performed with BAEC exactly as reported by Liaw et al. (20). Blocking actions of monoclonal antibodies were evaluated in migration experiments with HUVEC using transwell dishes as described previously (21). Del1 (100 nm) was employed in assays with 50 μg/ml anti-αvβ3 (clone LM609), 25–50 μg/ml anti-αvβ5 (clone PIF6), 1:20 dilution anti-β1 (clone J1B1a), and 20–40 μg/ml anti-ε2 (JBS2). Receptor blocking antibodies and cells were applied to the wells simultaneously. Each experiment was performed in quadruplicate and repeated at least three times.

**Immunofluorescence Analysis—**Coverslips were coated overnight at 4 °C with the adhesive substrates diluted in DPBS at a final concentration of 100 μg/ml washed 3 times with DPBS, and blocked with 10 mg/ml BSA in DPBS for 3 h at 37 °C. After growing cells on the coverslips for 3 h, they were fixed with 4% paraformaldehyde at room temperature for 15 min, permeabilized with acetone for 3 min, and washed 3 times with DPBS and nonspecific sites were blocked with 10 mg/ml BSA and allowed to stand for 20 min at 37 °C. Approximately 1.5–2×10^4 cells were applied to each dish and allowed to adhere. After 30, 60, or 120 min at 37 °C, cells were lysed with ice-cold RIPA lysis buffer or MAPK buffer (22). One group of serum-starved BAEC were washed with DPBS and lysed in the dish, and referred to as attached cells.

**Cell Culture—**Bovine aortic endothelial cells (BAEC) and human umbilical vein endothelial cells (HUVEC) were a kind gift from Dr. D. Vaughan (Vanderbilt University, Nashville, TN). BAEC were maintained in complete DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. HUVEC were maintained in EGM medium (Clonetics, San Diego, CA) supplemented with 20% fetal bovine serum and growth factors. Cells were cultured on 1% gelatin-coated tissue culture dishes and employed in experiments between passages 4–7.

**Adhesion Assays—**Test adhesive substrates were diluted in DPBS to 4% with the adhesive substrates diluted in DPBS at a final concentration of 100 μg/ml washing 3 times with DPBS and nonspecific sites were blocked with 10 mg/ml BSA in DMEM for 3 h at 37 °C. BAEC were harvested as described for the adhesion assay, suspended in DMEM containing 1 mg/ml BSA and allowed to stand for 20 min at 37 °C. Approximately 1–2×10^4 cells were applied to each dish and allowed to adhere. After 30, 60, or 120 min at 37 °C, cells were lysed with ice-cold RIPA lysis buffer or MAPK buffer (22). One group of serum-starved BAEC were washed with DPBS and lysed in the dish, and referred to as attached cells.
membranes. Immunoprecipitated tyrosine-phosphorylated proteins were detected by incubating with anti-phosphotyrosine monoclonal antibody at 1:1000 dilution. At a similar dilution polyclonal anti-p125FAK antibody was used to detect immunoprecipitated p125FAK. To verify equivalent amounts of p125FAK between samples, p125FAK immunoprecipitates were analyzed by Western blotting with polyclonal Del1 antisera. In both Del1 MJR and Del1 RAD fractions, the antibody recognizes bands migrating at 52 kDa (Fig. 1A and B), which are predicted sizes for Del1 MJR protein. The Del1 MNR isoform of Del1 was detected by the antibody at the expected size of 52 kDa, while the MNR form of Del1 was detected by the antibody at the expected size of 52-56 kDa, which is the predicted size for Del1 MJR protein. The Del1 MNR isoform of Del1 was detected by the antibody at the expected size of 52 kDa, while the MNR form of Del1 was detected by the antibody at the expected size of 52-56 kDa, which is the predicted size for Del1 MJR protein.

For MAPK experiments, cell lysates were prepared in the MAPK lysis buffer as described above in a total volume of 100 μl. Approximately 5–10% of the total lysates were resolved on a 12.5% SDS-PAGE and subsequently subjected to Western blotting as above. An anti-phospho-specific MAPK antibody that detects p42 and p44 MAPK only when catalytically activated by phosphorylation at Tyr204 and Thr202 (New England Biolabs) was used as an immunoprobe, followed by a secondary anti-rabbit horseradish peroxidase-conjugated immunoglobulin. Total MAPK protein was evaluated by probing the Western blots with a MAPK monoclonal antibody. For evaluation of phosphorylation of p125FAK, identical techniques were employed as for p125FAK and MAPK, except immunoprecipitation was performed with an anti-Shc antibody, and evaluated on Western blot with antibodies to phosphotyrosine and Shc (23).

RESULTS

**Recombinant Del1 Mediates Endothelial Cell Attachment in an RGD-dependent Manner**—Recombinant Del1 MJR, Del1 MNR, and Del1 RAD proteins were expressed in Sf9 insect cells, purified on a nickel column, and fractions were analyzed by SDS-PAGE and Western blotting with polyclonal Del1 antisera. Both Del1 MJR and Del1 RAD mutant proteins migrated at the expected size of 52 kDa, while the MNR form resolved into multiple bands at approximately 25 kDa (Fig. 1, A and B). Multiple bands observed with electrophoresis of the MNR protein may represent variation in glycosylation, other post-translational modification, or minor proteolytic degradation. Preimmune sera failed to recognize any of the recombinant forms of Del1 protein (data not shown). BAEC and HUVEC adhered to wells coated with recombinant bacterial and baculovirus forms of Del1, VN, and FN in a dose-dependent manner (Fig. 1C and data not shown). VN and FN were used as positive control adhesive substrates, based on earlier reports of their ability to promote endothelial cell adhesion (25). Dose-dependent adhesion was observed when coating solutions containing up to 100 nM of either of the two Del1 proteins were used, and half-maximal effect was observed using solutions containing 7 nM Del1, 10 nM VN, and 10 nM FN. Cell adhesion to Del1 was thus comparable to that observed with VN and FN substrates.

To investigate the role of the RGD motif in cell adhesion, site-directed mutagenesis was employed to construct a cDNA encoding a form of Del1 MJR with an RGD motif in the B loop of EGF repeat 2, instead of RGD. This mutant, Del1 RAD, was compared with the wild-type Del1 MJR, and the truncated protein that is encoded by the less common alternative Del1 transcript, Del1 MNR. The Del1 MNR protein contains the same signal sequence and three EGF-like regions as Del1 MJR, but has only a small portion of the amino-terminal discudin I-like domain (8). High titer baculovirus encoding these three proteins were generated and used to produce protein in Sf9...
cells, and recombinant proteins compared for their ability to mediate endothelial cell attachment (Fig. 1, A and D). Adhesion of BAEC to all the three forms of Del1 at 1, 10, 100, and 500 nM final coating concentrations were compared (Fig. 1 D). Both Del1 MJR and Del1 MNR showed a dose-dependent increase in attachment of BAEC, with a half-maximal effect for Del1 MJR at 7 nM. In contrast, the RAD mutant form of Del1 MJR supported cell attachment poorly, suggesting that the RGD motif in Del1 protein plays an important role in endothelial cell adhesion.

**Protein Competition Studies Suggest Adhesion to Del1 Is Integrin-mediated**—The effect of soluble Del1 on the adhesion of BAEC to extracellular matrix proteins was investigated. Microtiter wells were coated overnight with the test ligands at a final concentration of 100 nM, and cells were preincubated in solutions containing 1, 10, 100, and 250 nM Del1 for 30 min at 37 °C before applying them to the wells. Del1 protein was an effective competitor of BAEC adhesion to VN, with 50% inhibition obtained at 100 nM soluble Del1 protein (Fig. 2 A). Dose-dependent inhibition of BAEC adhesion to FN was also observed, with a maximum of 40% at 250 nM final concentration of soluble Del1. These data suggest that Del1 is capable of blocking a significant portion of the integrin-mediated endothelial cell attachment to VN and FN, but that Del1 interacts with only a subset of the receptors for each of these ligands.

In a similar series of experiments, the ability of soluble VN and FN to compete for cell adhesion to Del1 was investigated. The ability of BAEC to adhere to microtiter wells coated with 100 nM final coating concentration of Del1 in the presence of competing soluble adhesive ligands 10, 100, and 250 nM soluble VN and FN. A dose-dependent inhibition of BAEC adhesion to Del1 was observed (Fig. 2 B). Interestingly, an equivalent amount of inhibition was seen with both VN and FN, suggesting that Del1 may employ integrin receptors that are recognized by both VN and FN.

**RGD Peptide Competition Studies Confirm Adhesion to Del1 Is Integrin-mediated**—In peptide blocking studies, BAEC adhesion to Del1 at 10 nM coating concentration was inhibited with 100 μM GRGDSP hexamer peptide, with significant inhibition seen with as low as 10 μM peptide (Fig. 3 A). This inhibition of cell attachment is similar to that seen with RGD peptides competing for attachment to FN and VN substrates (Fig. 3 B, and data not shown). In contrast, even high concentration (500 μM final) of the control GRGESP peptides showed no significant inhibition. The concentrations of competing peptides used in these adhesion assays are typical for RGD-containing peptides used to compete other RGD-dependent binding activities (26). Synthetic peptides corresponding to the RGD sequence in Del1 showed similar dose-dependent inhibition of adhesion of BAEC to Del1 protein, with approximately 50% inhibition achieved with 100 μM peptide. The RGD-Del1 peptide could also compete for attachment of BAEC to VN and FN substrates in a similar adhesion assay (data not shown).

Experiments were also conducted with a peptide containing the penicillamine motif, Gly-Pen-Gly-Arg-Gly-Asp-Ser-Pro-Cys-Ala, which has been shown to have some specificity for...
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Fig. 4. Del1 mediates endothelial cell attachment and migration through ligation of specific vitronectin receptors. A, effects of integrin receptor blocking antibodies on HUVEC adhesion to Del1 were investigated. Approximately 75% inhibition was achieved with the αvβ3 receptor antibody. The antibody against the αvβ5 receptor and specifically the αv receptor subunit showed approximately 25% inhibition of cell adhesion, which was somewhat less than the 50% inhibition seen toward cell adhesion to VN. Antibodies to the β1 and α6 receptor subunits failed to inhibit adhesion. B, Del1 stimulates endothelial cell migration via a chemotactic mechanism. Migration of BAEC was evaluated after 6 h in a modified Boyden chamber assay, revealing the greatest migration when protein was in the bottom chamber only, consistent with a chemotactic mechanism. C, blocking antibodies were included in migration assays with HUVEC, and migration evaluated for Del1 and VN. Antibodies to αvβ3 blocked approximately 50% of the migration of the endothelial cells on both Del1 and VN. Antibodies to αvβ1 and αvβ3 failed to inhibit Del1-mediated endothelial cell migration.

Integrin receptors containing the αv subunit (26). Del1, VN, and FN were evaluated at 100 nM coating concentration, with peptides at 250 μM. The penicillamine containing RGD peptide strongly inhibited BAEC adhesion to both Del1 and VN, suggesting these cells use similar αv receptors to attach to Del1 and VN (Fig. 3B). RGD and control RGE peptides included in these experiments showed that inhibition of endothelial cell attachment by RGD peptides was equivalent for these two proteins.

Integrin Receptor Blocking Antibodies Suggest Del1 Binds αvβ3—To further define the integrin receptors involved in endothelial cell adhesion, blocking studies were performed with HUVEC and well characterized monoclonal antibodies. The monoclonal antibody against αvβ3 (LM609) routinely blocked 75% HUVEC adhesion to Del1, and this was comparable to results seen with VN (Fig. 4A). Interestingly, blocking antibodies that recognize another VN receptor, anti-αvβ5 (P1F6), consistently showed less blocking activity for cells on Del1, suggesting a lower affinity interaction with this receptor. Antibodies to the α6 subunit had no blocking activity associated with Del1 adhesion, but inhibited adhesion to laminin by approximately 80% (data not shown). Monoclonal antibodies to the β1 integrin subunit (clone JB1a) also failed to show significant inhibition. Taken together, these data suggest that Del1 mediates endothelial cell attachment primarily through molecular interaction with the αvβ3 receptor VN receptor, and to a lesser extent with the αvβ5 VN receptor. Additional receptors, such as αvβ1 might also be involved.

Del1 Stimulates Endothelial Cell Migration—The ability of Del1 to mediate endothelial cell migration was evaluated with studies using a Boyden-type chamber assay (19). Del1 stimulated a dose-dependent migration of BAEC with half-maximal effect observed at 75 nM (data not shown). To test whether the nature of this migration was chemotactic or chemokinetic, the location of Del1 protein (100 nM final concentration) in the migration chamber was varied. A chemotactic effect was observed, since a gradient of Del1 (bottom only) was required for maximal migratory activity (Fig. 4B). VN at the same concentration was also chemotactic for endothelial cells in this assay, in agreement with earlier results (21). Specificity of the migratory effect on HUVEC was tested with blocking antibodies to integrin receptors (Fig. 4C). Approximately half of the chemotactic migration in response to Del1 was inhibited by blocking antibodies against αvβ3. Antibodies to the αvβ5 vitronectin receptor, and the α2β1 collagen/laminin receptor did not alter HUVEC migration.

Endothelial Cells Form Focal Contacts on Del1—Endothelial cells attached and spread on VN and Del1 in a similar fashion, although the process was slower on Del1. Cells attached and spread as early as 30 min on VN, but required 60 min for this process on Del1. BAEC and HUVEC in the BSA-coated wells remained rounded even after 120 min (data not shown). Active protein synthesis was not required for the attachment of either endothelial cell type to Del1 since pretreatment of cells with 25 μg/ml cycloheximide for 2 h and maintenance of the drug during the entire assay period did not decrease the total number of attached cells.

To investigate whether cell attachment and spreading on Del1 is associated with cytoskeletal and signaling events associated with integrin activation, we performed a number of immunofluorescent studies with cultured endothelial cells. BAEC were allowed to adhere and spread on Del1-coated coverslips for 3 h, fixed, and stained with fluorescein isothiocyanate-conjugated phallolidin or monoclonal antibodies to vinculin, talin, or the β3 integrin subunit. Labeling with phallolidin revealed actin polymerization and filament formation in these cells plated on Del1, similar to that seen in endothelial cells plated on VN (Fig. 5). Labeling with the vinculin and talin antibodies showed that the cells had formed focal contacts on Del1, as demonstrated by the localization of vinculin and talin in discrete contact areas within the cell. The attachment of BAEC to Del1 also led to the clustering of integrin receptors containing the β3 subunit, presumably αvβ3, as demonstrated by a punctate staining pattern similar to that seen with the vinculin and talin antibodies (Fig. 5). Cells were also stained with normal mouse IgG as a control or without the primary antibody and no specific immunofluorescence was observed (data not shown). Also, no difference in the staining was observed when cells were treated with cycloheximide.

Integrin signaling has been linked to phosphorylation of proteins in the focal adhesions associated with integrin clustering. As a first step to define the cellular response to ligation of integrin receptors with Del1, immunofluorescent staining experiments were performed with cultured BAEC and anti-phosphotyrosine antibodies. In these studies, labeling was seen in the focal contacts in the endothelial cells adhered to Del1, similar to the pattern that was detected with cells plated on VN (Fig. 5).

Adhesion and Spreading of BAEC on Del1 Induces Tyrosine
Phosphorylation of p125FAK, MAPK, and Shc—p125FAK undergoes phosphorylation in focal contacts, and has been implicated in integrin signaling pathways (27). To investigate whether adhesion to Del1 is associated with phosphorylation of p125FAK, immunoprecipitation and blotting experiments were conducted. Serum-starved BAEC were allowed to attach and spread on dishes coated with Del1 or VN, and harvested after 30, 60, or 120 min at 37 °C. Cells were lysed, immunoprecipitated, and subjected to Western analysis with antibodies to phosphotyrosine and p125FAK. A time-dependent increase in phosphorylation of one or more 120-kDa proteins occurred on both the substrates, with a maximum level reached at 60 min for VN (Fig. 6A, arrowhead). Phosphorylation in cells adherent to Del1 gave a similar but somewhat delayed increase. Stripping and reprobing the blot with anti-p125FAK showed that one of the 120-kDa phosphorylated proteins corresponded to p125FAK (Fig. 6B). Total lysates immunoprecipitated with anti-p125FAK and probed on Western blots with the same antibody showed that equal amounts of this protein were present under all conditions of the experiment (Fig. 6C).

To investigate MAPK activation, lysates were prepared from attached and detached cells, as outlined for p125FAK experiments, and analyzed by Western blotting with a phospho-specific MAPK antibody (Fig. 6, D and E). Activation of p44 and p42 MAPK occurs through phosphorylation of specific threonine and tyrosine residues (202 and 204 of the human MAPK (ERK 1)) at the sequence T*EY* by a single upstream MAPK kinase, MEK (28, 29). The phospho-specific MAPK antibody detects p42 and p44 MAPK (ERK1 and ERK2) only when activated by tyrosine phosphorylation on these residues. In this experiment, MAPK was phosphorylated in the attached condition, and revealed a rapid increase in phosphorylation after cells attached to Del1 and VN. Increased phosphorylation was observed by 30 min after the cells spread on either Del1 and VN substrates, and was sustained (Fig. 6D). Probing Western blots with a polyclonal anti-MAPK antiserum showed that equal amounts of MAPK were present under all conditions of the experiment (Fig. 6E).

The adapter protein Shc has been implicated in functions mediated by αvβ3, thus additional experiments evaluated the phosphorylation of Shc in endothelial cells on recombinant Del1 and VN (30). Experiments were performed as for p125FAK and MAPK, with immunoprecipitations from cell lysates being performed with an anti-Shc antibody. Precipitated protein was evaluated on Western blot for level of phosphorylation by probing with a phosphotyrosine antibody. These data revealed a time-dependent increase in phosphorylation of Shc in cells on VN and Del1, with a peak at approximately 60 min (Fig. 6F). Equal amounts of Shc protein were present within the experimental conditions (Fig. 6G).

Del1 Is Angiogenic in the in Ovo Chick CAM Assay—Baculovirus Del1 MJR was evaluated in the in ovo CAM assay at 100 nM and 1 μM, and found to stimulate angiogenesis at both concentrations (Fig. 7, A, C, and D). At the higher concentration baculovirus Del1 MJR produced an angiogenic response similar to the maximal bFGF response. Mammalian cell expressed Del1 MJR was also angiogenic, and was significantly more potent, achieving maximal levels of angiogenesis at 100 nM (Fig. 7B). This difference in early batches of protein most likely reflected technical difficulty with the purification rather than differences in folding or glycosylation, since subsequent purifications have yielded baculovirus protein with potency similar to the mammalian expressed protein (data not shown).

In experiments where Del1 MJR was added to the same filter disc 1 day after bFGF, it did not inhibit the angiogenic actions of this growth factor, and also did not add to the angiogenesis that was observed with bFGF alone (Fig. 7B). When applied to the CAM in an identical fashion at 1 μM concentration, VN and FN were not angiogenic (Fig. 7C).

The role of integrin binding in Del1-mediated angiogenesis was evaluated in two ways. First, an αvβ3 monoclonal antibody with blocking activity was introduced into the CAM assay along with Del1 MJR protein, where it was able to abrogate completely the angiogenic response to baculovirus Del1 protein (Fig. 7, A and D). A control antibody of the same isotype, antibody W632 directed against an myosin heavy chain class I antigen, had no effect on angiogenesis. In a separate series of experiments, baculovirus Del1 MJR protein with an RGD → RAD mutation was compared with the wild-type Del1 MJR protein in CAM assays (Fig. 7, A and D). The RAD mutant protein had only minimal angiogenic activity. These data strongly suggest that the angiogenic actions of Del1 require endothelial cell integrin activation.

**DISCUSSION**

The pattern of expression of Del1 in embryogenesis, and the limited functional data available from initial experiments have provided a complex picture regarding the role of Del1 in vascular development (8). The transient expression pattern of this protein in the embryonic endothelium, and the microvascular angiogenic aspect of the response observed in CAM assays employing tumor cells expressing Del1, suggested an angiogenic or angiogenesis supporting role during vascular formation. However, persistent expression of Del1 in endothelial cells not associated with vascular formation, and expression by non-endothelial cells known to produce inhibitors of blood vessel formation, suggested an inhibitory function. The ability of Del1 to inhibit in vitro tube formation by cultured yolk sac cells, and the apparent loss of large patterned vessels in the CAM was consistent with an inhibitory function or a role in the complex...
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Del1 is a functional integrin ligand. Cell adhesion to Del1 is comparable to adhesion on similar concentrations of VN and FN, and Del1 mediates endothelial cell migration similar to that seen on VN. These activities are mediated primarily through Del1 binding the αvβ3 VN receptor, and in part through the αvβ5 VN receptor. Endothelial cells attach and spread on Del1, and this is associated with the formation of focal contacts that include vinculin and talin. Proteins in the focal contacts, including p125FAK, show increased phosphorylation when endothelial cells attach and spread on Del1. Del1-mediated integrin signaling is suggested by the time-dependent phosphorylation of Shc and MAPK in endothelial cells on Del1. Thus, in all aspects investigated thus far, Del1 appears to function as a classical integrin ligand. Of course, one unique feature of Del1-integrin interaction is the cell-specific and potential autocrine nature of its function, which result from the temporal endothelial cell restricted pattern of expression of Del1.

Given these data supporting a high affinity productive interaction of Del1 with endothelial cell integrins, it was important to investigate again the functional profile of Del1 proteins in the CAM assay. Also, the use of recombinant affinity purified baculovirus protein, in comparison to the transfected cell lines used as a source for Del1 in the initial studies, was felt likely to give more easily interpreted results. Surprisingly, in an in ovo model of the CAM assay that has been employed extensively in the laboratory of one of the investigators, the Del1 MJR isoform was found to be a potent angiogenic factor (24). Indeed, quantitation of an average number of vessel branch points indicated that Del1 MJR was equally active as bFGF in this angiogenesis model. While the initial preparations of baculovirus protein were less active on a molar basis than bFGF, such as in Fig. 7, later preparations of protein were as active as FGF on a molar basis (data not shown). There was no evidence of the loss of vascular pattern or a decrease in the number of larger vessels, such as that observed in the initial CAM assays employing tumor cells expressing Del1 MJR. To further evaluate this difference in functional profile, to obviate the possibility that the angiogenic activity of recombinant protein might represent a contaminating baculovirus or insect cell protein, Del1 MJR was expressed in eukaryotic cells. Human osteosarcoma cells transfected with a Del1 MJR expression construct were employed in culture as a source of Del1 MJR, and protein was purified from cultured supernatant with a 7-step column purification strategy. This protein was also found to be highly angiogenic in the in ovo chick CAM assay (Fig. 7).

There are multiple possible reasons for the differences observed in these CAM assays employing purified protein versus those employing transfected tumor cells as a source of Del1. First, the complex vascular reorganization observed in the CAM assay employing tumor cells could represent the independent activity of factors produced by the cultured cells, or the actions of such tumor cell-derived factors in combination with Del1. Alternatively, the cultured cells might be stimulated by Del1 to produce a factor or factors that alone or in combination with Del1 produce the observed vascular reorganization. It is also possible that the observed differences simply represent differences in the amount of Del1 protein provided to the CAM. It has been established that thrombospondin, for instance, can produce differing effects on the developing vasculature, depending on the concentration of protein that is provided to the endothelial cells (33). However, this latter explanation would seem unlikely since a dose-response experiment was performed

process of vascular remodeling (8). Regarding this second possibility, it was difficult to understand how Del1 might mediate such a function through its interactions with the αvβ3 receptor. All evidence to date has indicated that signaling through ligation of this receptor is essential for supporting angiogenesis (6, 31, 32). To reconcile an inhibitory functional profile with the angiogenic activity of recombinant protein might represent a contaminating baculovirus or insect cell protein, Del1 MJR was expressed in eukaryotic cells. Human osteosarcoma cells transfected with a Del1 MJR expression construct were employed in culture as a source of Del1 MJR, and protein was purified from cultured supernatant with a 7-step column purification strategy. This protein was also found to be highly angiogenic in the in ovo chick CAM assay (Fig. 7).

There are multiple possible reasons for the differences observed in these CAM assays employing purified protein versus those employing transfected tumor cells as a source of Del1. First, the complex vascular reorganization observed in the CAM assay employing tumor cells could represent the independent activity of factors produced by the cultured cells, or the actions of such tumor cell-derived factors in combination with Del1. Alternatively, the cultured cells might be stimulated by Del1 to produce a factor or factors that alone or in combination with Del1 produce the observed vascular reorganization. It is also possible that the observed differences simply represent differences in the amount of Del1 protein provided to the CAM. It has been established that thrombospondin, for instance, can produce differing effects on the developing vasculature, depending on the concentration of protein that is provided to the endothelial cells (33). However, this latter explanation would seem unlikely since a dose-response experiment was performed
with each batch of purified protein.

The finding that Del1 is angiogenic is surprising and intriguing. While significant evidence suggests that integrin signaling is required for angiogenesis, by supplying survival signals that prevent apoptosis, there is no evidence that integrin ligands can initiate the angiogenic process. Indeed, experiments presented here show that vitronectin and fibronectin are not angiogenic in the CAM assay. These data suggest two hypotheses. One possibility is that Del1 can initiate angiogenesis through a unique molecular signaling pathway. The requirement for αvβ3 receptor activation indicates that integrin signaling is a critical component of the angiogenic response, and is consistent with this possibility. However, all data presented here suggests that Del1 signaling is not different from that of other matrix ligands such as vitronectin and fibronectin. Currently, little is known regarding angiogenic signaling pathways, making further study of this hypothesis difficult.

A second hypothesis is that Del1 stimulates angiogenesis through a second signaling pathway. This activation pathway might promote endothelial cell division as seen with angiogenic growth factors, or inhibit the differentiation of endothelial cells so that they can respond to local growth factor signals. The EGF repeats of Del1 have amino acid similarity with the notch receptors and their ligands, and interaction of these proteins are known to inhibit terminal differentiation of a wide variety of cell types. There is an endothelial cell-specific form of notch, Notch4, and preliminary studies suggest that notch signaling can regulate the endothelial cell phenotype (34, 35). Del1 signaling, through a notch-related receptor, might function to promote an undifferentiated phenotype, with continued cell division being a component of that program. These cellular functions would be appropriate for the endothelial cell involved in angiogenesis. In the context of this hypothesis, data presented here indicating that Del1 angiogenic activity requires αvβ3 activation would suggest that the receptor mediating this second signaling pathway must be coordinately activated in

FIG. 7. Del1 is angiogenic in the chick CAM assay, and this activity requires integrin signaling. A, baculovirus Del1 MJR (Del1) and Del1 MJR RGD → RAD mutant (Del1 RAD) proteins at 1 μM (2 μg total), bFGF at 59 nM (30 ng total), or phosphate-buffered saline (PBS) were applied to the chick chorioallantoic membrane and their effects on vascular development evaluated. In some experiments, the αvβ3 blocking antibody LM609 (Del1+LM609) or an unrelated control antibody W632 (Del1+W632) were added after 1 day, and their ability to modify the angiogenic response evaluated. B, quantitative comparison of angiogenic activity of mammalian murine Del1 protein at 100 nM and bFGF at 59 nM. Maximal angiogenic response achieved with Del1 was similar to that seen with the maximal bFGF dose employed, and Del1 did not augment or inhibit the bFGF response. C, VN and FN at 1 μM were compared with baculovirus Del1 MJR at 1 μM, and found to not stimulate angiogenesis in the CAM assay. D, quantitation of CAM data with baculovirus protein indicated that integrin signaling is required for angiogenic activity. Recombinant baculovirus Del1 MJR protein was inactive in the presence of the αvβ3 blocking antibody LM609, but was not affected by the negative control antibody W632. Also, Del1 RAD had significantly reduced angiogenic activity.
conjunction with αvβ3 ligation. While such interaction of an integrin receptor and a second cell surface receptor has not been studied in detail, αvβ3 has been shown to interact with a thrombospondin-binding protein (36, 37).

Despite the potential complexity of its molecular actions, these data provide the basis for a more complete understanding of the role of Del1 in vascular development. Through its interaction with integrin receptors, Del1 may provide the endothelial cell an autocrine pathway for regulating its own migration and response to growth and differentiation factors. Endothelial cells in the embryo migrate widely, and migration is a requisite and response to growth factor-pathway might also have a critical role in protecting the endothelial cell from apoptosis as it migrates through growth factor-rich environments in the embryo. The αvβ3 receptor has a unique role in supporting division of the endothelial cell by protecting it from apoptosis, and this receptor has been found to be essential for embryonic vascular formation (6, 39). In embryogenesis and other situations where the endothelial cell expresses Del1, it may be the primary ligand that supports the function of this receptor. If Del1 has the capacity to activate a second pathway that initiates angiogenesis, in a fashion similar to angiogenic growth factors, this would make Del1 a uniquely potent activator of vascular growth.

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