Heparin-II Domain of Fibronectin Is a Vascular Endothelial Growth Factor–Binding Domain

Enhancement of VEGF Biological Activity by a Singular Growth Factor/Matrix Protein Synergism

Errol S. Wijelath, Salman Rahman, Mayumi Namekata, Jacqueline Murray, Tomoaki Nishimura, Zohreh Mostafavi-Pour, Yatin Patel, Yasuo Suda, Martin J. Humphries, Michael Sobel

Abstract—We describe extracellular interactions between fibronectin (Fn) and vascular endothelial growth factor (VEGF) that influence integrin-growth factor receptor crosstalk and cellular responses. In previous work, we found that VEGF bound specifically to fibronectin (Fn) but not vitronectin or collagens. Herein we report that VEGF binds to the heparin-II domain of Fn and that the cell-binding and VEGF-binding domains of Fn, when physically linked, are necessary and sufficient to promote VEGF-induced endothelial cell proliferation, migration, and Erk activation. Using recombinant Fn domains, the C-terminal heparin-II domain of Fn (type III repeats 13 to 14) was identified as a key VEGF-binding site. Mutation of the heparin-binding residues on FnIII13–14 abolished VEGF binding, and peptides corresponding to the heparin-binding sequences in FnIII13–14 inhibited VEGF binding to Fn. Fn fragments containing both the αβ1 integrin-binding domain (III 9 to 10) and the VEGF-binding domain (III 13 to 14) significantly enhanced VEGF-induced EC migration and proliferation and induced strong phosphorylation of the VEGF receptor and Erk. Neither the cell-binding or VEGF-binding fragment of Fn alone had comparable VEGF-promoting effects. These results suggest that the mechanism of VEGF/Fn synergism is mediated extracellularly by the formation of a novel VEGF/Fn complex requiring both the cell-binding and VEGF-binding domains linked in a single molecular unit. These data also highlight a new function for the Fn C-terminal heparin-binding domain that may have important implications for angiogenesis and tumor growth. (Circ Res. 2006;99:853-860.)

Key Words: endothelial cell differentiation ■ endothelial cell growth ■ endothelial cells ■ fibronectin ■ integrins ■ signal transduction ■ vascular endothelial growth factor ■ vascular endothelial growth factor receptors

Fibronectin (Fn) and vascular endothelial growth factor (VEGF or VEGF-A) are key regulators of blood vessel growth.1–3 Gene deletion studies have demonstrated that both Fn and VEGF, and their cognate receptors αβ1 and VEGF receptor-2 (VEGFR-2), are critical for vascular development.4–9 During vascular growth, endothelial cells (ECs) are recruited into a tightly controlled program of cell activation, gene expression, adhesion, motility, and proliferation.10,11 Crosstalk between integrins and growth factor receptors (receptor tyrosine kinases, RTKs) is a key part of this control process.12–16 EC responses to growth factors like VEGF are modulated by the outside-in signals conveyed from integrins, reflecting the conditions of the extracellular milieu. Hence, the local mixture of extracellular matrix (ECM) proteins as well as the type and density of integrins expressed by the cell may modulate its responses to growth factors. Integrin engagement by a specific matrix protein can transactivate VEGF receptors, as shown by Groopman and colleagues for fibronectin (Fn) and VEGF receptor-3, via αβ17 and by Moro et al for epidermal growth factor receptor.18 Others have shown that β3 activation by Fn can modulate VEGF responses,19 and that β1 activation may increase VEGF production in tumor cells.20 Conversely, occupancy of VEGFR-2 by VEGF can activate integrins. Byzova et al have shown that VEGF activates both αβ, and αβ1 on ECs.21 Finally, integrins and receptor tyrosine kinases (RTKs) share a number of important signaling molecules, including Src, FAK, and phosphatidylinositol 3-kinase (PI3K).13 Our previous work has focused on the specificity of interactions between Fn and VEGF, and their cognate receptors, αβ1 and VEGFR-2.22,23 We found VEGF bound to Fn and that platelets secreted VEGF/Fn complexes. In the presence of the
ligands VEGF and Fn, their corresponding receptors coinmunoprecipitated. Fn, but not vitronectin, induced sustained mitogen-activated protein kinase (MAPK) activation in response to VEGF. Thus we predicted that the VEGF-enhancing activities of Fn were attributable, in part, to its binding to VEGF.

This present study was undertaken to more precisely map the binding site(s) for VEGF within the C-terminal regions of Fn and to determine the mechanisms of Fn-induced enhancement of VEGF activity. Using recombinant Fn fragments, and synthetic peptides, we have mapped the VEGF-binding site to the established heparin-II (Hep-II) site of Fn type III modules 13 to 14.24,25 This VEGF-binding domain of Fn was found to be necessary but, by itself, was not sufficient for the promotion of VEGF biological activity. Only bivalent Fn constructs encompassing both the αβi and VEGF-binding domains significantly promoted endothelial migration, proliferation, and sustained Erk phosphorylation. Purely costimulatory signaling (via occupancy of integrin and VEGFR-2 receptors) was a weaker influence on VEGF activity, because mixtures of VEGF with monovalent Fn fragments containing cell- and VEGF-binding domains showed less migration, proliferation, and no late phase of Erk phosphorylation.

Materials and Methods

Cell Culture

Human umbilical vein ECs (HUVECs) (Cascade Biologics) were maintained in MCDB-131 growth medium (MCDB-131 medium containing 5% FBS, 2 ng/mL basic fibroblast growth factor [bFGF], 10 ng/mL VEGF, and 10 μg/mL heparin). HUVECs between passage 3 to 8 were used for experiments.

cDNA Construction

Human Fn cDNA was obtained by RT-PCR using human liver mRNA and sequenced to verify no base changes during PCR amplification. The Fn type III modules were included by PCR amplification using Fn cDNA as template. We used the following nomenclature for describing the recombinant Fn constructs:

- rFnIII9–10: indicates recombinant Fn protein encompassing type III repeats 9 to 10; rFnIII12–14: a single protein encompassing type III repeats 9 to 14, omitting 11. A rFnIII9–14 with the heparin-binding sites on FnIII domains 13 and 14 mutated (Arg or Lys → Ser, termed “gag AC”) was obtained as previously described.26 For details on the construction of the rFn plasmids and their expression and purification, please see the online data supplement, available at http://circres.ahajournals.org.

EC Migration Assay

EC chemotactic migration to VEGF (10 ng/mL; upper chamber) was studied in the presence of different rFn fragments (0.2 μmol/L; bottom chamber). Ninety-six well ChemoTx microtiter plates (NeuroProbes Inc) were used, as previously described.22,27 Positive and negative controls included native Fn, vitronectin, or albumin all at 10 μg/mL, in the place of the tested rFn fragments.

Endothelial Proliferation Assay

Early-passage HUVECs (3000 cells/well) in 96-well plates were cultured in MCDB-131 medium containing fibronectin-depleted FBS (0.25%). Cells were incubated with albumin, VEGF alone (10 ng/mL), or VEGF with native Fn (10 μg/mL) or rFn fragments (0.2 μmol/L) for 72 hours. Heparin was used at a final concentration of 1 μg/mL. Cell growth was determined by using CyQuant reagent (Molecular Probes) according to the instructions of the manufacturer.

Western Blot Analyses

For analysis of Erk phosphorylation, 5×10⁴ HUVECs were plated onto 60-mm culture dishes and incubated overnight in MCDB-131 growth medium. Cells were then washed twice and incubated for 4 hours in serum-free MCDB-131 medium. Cells were stimulated with VEGF (10 ng/mL) or VEGF with different Fn combinations. At different time points, cells were lysed and Erk phosphorylation detected as previously described.28 Signal densities were quantified by the NIH Image program, and the activation of phosphorylated protein was expressed as the fold increase over basal (unstimulated) levels, adjusted for total protein loading, measured from separate blots.

To measure VEGFR-2 phosphorylation, HUVECs (2×10⁸ cells) were cultured for 48 hours in MCDB-131 growth medium. Before assay, cultures were washed and replaced with serum-free MCDB-131 containing ITS supplement (BD Bioscience) and incubated for a further 4 hours. Culture plates were then stimulated with a low dose of VEGF (1 ng/mL) in the absence or presence of rFNIII9–10/12–14 for 2 minutes at 37°C. Cell lysates were incubated with anti–VEGFR-2 rabbit monoclonal antibodies (Cell Signaling) for 16 hours at 4°C and immune complexes precipitated with protein G sepharose. Protein samples were reduced, separated by Bis-Tris PAGE gels, transferred to polyvinyl difluoride (PVDF) membranes, probed with anti-phospho VEGFR-2 antibodies and signals developed by chemiluminescence. Blots were stripped and reprobed with anti–VEGFR-2.

125I-VEGF Solid-Phase Binding Assays

The measurement of direct binding of 125I-VEGF to Fn fragments was performed as described previously.22 For peptide competition assays, the same binding assay methods were used, with recombinant C-terminal rFn III9–10/12–14 or rFnIII12–14 immobilized on the plates.

Surface Plasmon Resonance Analysis

Surface plasmon resonance analysis (SPR) binding experiments were conducted to measure the equilibrium binding parameters between VEGF165 and native fibronectin or rFnIII9–10/12–14. The detailed methods are described elsewhere.29 In brief, a 2-channel SPR670M apparatus (Moritex Corp) was used with a running buffer of PBS, 0.05% Tween-20 at a flow rate of 15 μL/min at room temperature. The matrix proteins were immobilized on the gold chip using N-hydroxysuccinimidy coupling, sensograms recorded over a range of VEGF concentrations (25 to 800 μmol/L), and the kinetic binding parameters derived using the software of the manufacturer.

Statistical Analysis

Individual experiments were performed at least in triplicate, and the data presented are the means and SEM of at least 3 to 4 independent experiments, as noted. The Student’s t test was used to compare different treatments.

Results

Expression and Purification of Fibronectin Fragments

Figure 1 illustrates a schematic of the native fibronectin protein and the design of the recombinant fragments. Each protein was expressed and purified to homogeneity, as described in Materials and Methods. A Bis-Tris PAGE gel shows representative samples of the purified proteins.

VEGF Binding to Recombinant Fn Domains and Competition by Peptides

We first measured the concentration dependence of 125I-VEGF binding to different immobilized recombinant Fn fragments. These experiments were all conducted in the absence of exogenous heparin, indicating that heparin is not a
required cofactor for VEGF/Fn binding. In preliminary experiments, the actual quantity of experimental proteins deposited on the plate was measured by protein assay, confirming that the final density of coating was comparable for all test proteins. As shown in Figure 2, VEGF bound rFnIII_{12–14} and rFnIII_{8–14} in a concentration-dependent manner. Binding to rFnIII_{8–11} was negligible. In subsequent binding assays, a coating concentration of 0.2 μmol/L was used for all proteins. The binding of VEGF to individual rFnIII domains was then evaluated. Figure 3A shows that VEGF does not bind significantly to any of the individual type III domains studied but did bind to all fragments containing FnIII_{13–14} in continuity (ie, native Fn, rFn_{8–14}, rFn_{12–14}). Figure 3A and 3B shows that the linkage of the αβ integrin-binding domain with rFnIII_{12–14} (as in the constructs rFnIII_{8–14} or III_{9–10/12–14}) further enhanced VEGF binding when compared with rFnIII_{12–14} alone. The binding of VEGF to rFnIII_{8–14} and rFnIII_{9–10/12–14} when compared with native Fn was consistently higher, suggesting a higher affinity for these fragments. Surface plasmon resonance binding analysis was used to compare the VEGF affinities of rFnIII_{9–10/12–14} to native Fn. The rFnIII_{9–10/12–14} fragment had a 43% lower dissociation constant ($K_d$, 73.5 versus 129.3 nmol/L), a faster on-rate and a smaller off-rate (supplemental Table I). Because the region of FnIII_{12–14} is known to be an important heparin-binding region,^{24,25,30} we wanted to determine whether the key heparin-binding residues of FnIII_{12–14} were required for binding

Figure 1. Schematic diagram of the domain structure of human Fn showing type I, type II, type III and their functions. Also diagramed are the recombinant Fn type III domains used in this study, and on the left is a representative SDS-PAGE gel of purified fragments.

Figure 2. Concentration dependent $^{125}$I-VEGF binding to immobilized rFn fragments. $^{125}$I-VEGF was incubated for 2 hours on wells coated with increasing concentrations of rFn fragments. After washing, bound VEGF was eluted with 0.1 mol/L NaOH/1% SDS and radioactivity quantified in a gamma counter. Data are presented as the mean of triplicate determinations ± SEM.
VEGF. To that end, the construct rFnIII\(_{9-10/12-14}\)gagAC was prepared, in which the lysines and arginines of repeats 13 and 14 were mutated to serine, as previously described.\(^{26}\) Figure 3B shows that the loss of these heparin-binding residues completely abolished VEGF binding.

Peptides (listed in the Table) encompassing the native heparin-binding regions in FnIII\(_{13-14}\) were also used as soluble competitors of \(^{125}\)I-VEGF binding to immobilized rFnIII\(_{12-14}\) or rFnIII\(_{9-10/12-14}\). Two other peptides were also studied: a truncated version containing the putative key heparin-binding site of FnIII\(_{13}\), as well as a well-characterized heparin-binding site from the A1 domain of von Willebrand factor (vWF)\(^{31}\) (Table). The results of peptide competition against VEGF binding to either rFnIII\(_{12-14}\), or rFnIII\(_{9-10/12-14}\) substrates were not significantly different, so the data were pooled. The Table shows their estimated IC\(_{50}\) (concentration that inhibits VEGF binding by 50%), and Figure 4 shows their inhibition curves. The most potent peptide was a heparin-binding region in FnIII\(_{14}\) (core motif is PRAR). Second and third in rank order were the heparin-binding peptides derived from FnIII\(_{13}\) (core motif PRRAR) and FnIII\(_{14}\) (IYVIALKNNQKSEPLIGRKKT), respectively. Truncating the heparin-binding domain of FnIII\(_{13}\) eliminated its ability to compete with the immobilized Fn fragment for binding VEGF. Figure 4B correlates the potency of each peptide competitor with its total number of basic residues. The peptide charge accounts for some, but not all, of the apparent affinity of the peptides for VEGF. The most potent Fn peptide bears the same net charge as the vWF heparin-binding peptide but has an IC\(_{50}\) that is 600 times lower. The other 2 Fn peptides that share the PRRAR or PRAR motif possess the same number of heparin-binding residues but differ in affinity by 3.5-fold.

### Specificity of Fn Domains That Promote VEGF-Mediated EC Cell Migration and Proliferation

We hypothesized that the formation of a unique extracellular VEGF/Fn complex promotes VEGF biological activity, mediated in part by the bivalent capacity of Fn to simultaneously bind \(\alpha_\beta\) and VEGF. Accordingly, we measured the migration and proliferation of HUVECs in response to VEGF, in the presence of different native and recombinant matrix proteins. Figure 5A shows that there was minimal cellular migration toward VEGF in the absence of a matrix component, or with the addition of rFnIII\(_{9-10}\) alone. Most of the proteins containing an integrin-binding domain (eg, vitronectin, rFnIII\(_{9-10}\) and rFnIII\(_{9-10/12-14}\)gagAC) supported some migration to VEGF. In contrast, all bivalent matrices encompassing both the \(\alpha_\beta\) and the VEGF-binding domains (FnIII\(_{8-14}\) and FnIII\(_{9-10/12-14}\)) increased cell migration by 2- to 3-fold. To distinguish between simple costimulatory signaling through the independent ligands (ie, integrin and growth factor receptor), chemotactic migration to VEGF was also measured in the presence of equivalent concentrations of a mixture of the isolated domains of rFnIII\(_{9-10}\) and rFnIII\(_{9-12-14}\). This mixture did support a modicum of cell migration above baseline but did not increase migration to the extent induced by a bivalent Fn construct that linked both those domains.

We next examined whether rFn fragments promoted VEGF-induced HUVEC proliferation. As shown in Figure 5B, the extent of HUVEC growth in response to VEGF was enhanced in the presence of native Fn or rFnIII\(_{9-10/12-14}\), compared with the cell or VEGF-binding domains alone (rFnIII\(_{9-10}\) or rFnIII\(_{9-12-14}\)). Addition of heparin (1 \(\mu\)g/mL) to the VEGF/Fn complex further augmented proliferation to the same extent heparin augmented proliferation of VEGF alone. A mixture of cell-binding and VEGF-binding fragments of Fn

### Table: Relative Inhibition of \(^{125}\)I-VEGF\(_{165}\) Binding by Heparin Binding Peptides

<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptide Sequence</th>
<th>IC(_{50}) ((\mu)mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rFnIII(_{13})</td>
<td>1814 wspvrarrvttcttitcttetcctf</td>
<td>43</td>
</tr>
<tr>
<td>rFnIII(_{13}) (truncated)</td>
<td>1814 spprrav</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>rFnIII(_{14})</td>
<td>1926 prartgyiylkekpgsppreprpgv</td>
<td>0.4</td>
</tr>
<tr>
<td>rFnIII(_{14})</td>
<td>1971 iyvialknqksepligrkkt</td>
<td>150</td>
</tr>
<tr>
<td>von Willebrand factor</td>
<td>568 kdkrselnriqsvq</td>
<td>250</td>
</tr>
</tbody>
</table>
(at the same concentrations, 10 μg/mL) showed less increase in proliferation compared with the divalent fragment.

Modulation of VEGF-Mediated Signaling by Specific rFn Domains
Previously, our laboratory reported that VEGF/Fn complexes prolonged cell signaling induced by VEGF alone, suggesting that such extracellular complexes may influence the magnitude and duration of cell signaling. Therefore, we examined the time course and sensitivity of Erk signaling to VEGF and different Fn domains. As shown in Figure 6, VEGF combined with native Fn produced a higher early (5 minutes) peak in phosphorylation, as well as more sustained activation at 1.5 to 3 hours. In comparison with native Fn, the rFnIII<sub>9–10/12–14</sub> induced an even higher early peak and a second peak of Erk activation at approximately 1.5 hour. A mixture of rFnIII<sub>9–10</sub> and rFnIII<sub>12–14</sub> (at the same individual concentrations as other fragments tested) did not induce any late Erk activation. When tested individually, the cell binding (rFnIII<sub>9–10</sub>) or VEGF-binding domains (rFnIII<sub>12–14</sub>) did not induce any late Erk activation. When tested individually, the cell binding (rFnIII<sub>9–10</sub>) or VEGF-binding domains (rFnIII<sub>12–14</sub>) did not induce any late Erk activation. The divalent recombinant Fn fragment rFnIII<sub>9–10/12–14</sub> significantly enhanced VEGF-induced Erk phosphorylation even at very low VEGF concentrations, between 0.1 to 0.5 ng/mL (Figure 7A and 7B).

Discussion
Based on our previous studies, we hypothesized that Fn may be a unique biological partner with VEGF: when Fn complexes with VEGF, their coordinated binding to their cognate receptors enhance the specific cellular responses to VEGF. These extracellular events might be an important step in modulating the complex signaling pathways that lead from receptor ligation to cellular response. In this report we have physically mapped a key Fn-binding site for VEGF to the fibronectin C-terminal domain within type III repeats 13 to 14 (the Hep-II domain). Previous studies have established this site as a heparin-binding domain. The Hep-II site is a major syndecan-binding site that plays a role in focal adhesion and stress fiber formation. This report reveals a novel function for the Hep-II region as a site for VEGF binding, as well as a modulator of VEGF activity. Narrowing the binding site further, we observed that a peptide obtained from the N-terminal part of FnIII<sub>14</sub> was most effective at blocking VEGF binding, although other peptides representing heparin-binding sites were also inhibitory. Also, the heparin-binding properties of this Fn domain accounted for a significant portion of VEGF affinity. A heparin-mutant fragment did not bind VEGF, nor did it promote the biological activities of VEGF.

The second important finding from these experiments concerns the mechanism(s) of biological synergism between VEGF and Fn. We found that the VEGF-binding domain of Fn alone was not sufficient to enhance VEGF-mediated cell migration, proliferation, or growth factor signaling, even though it avidly bound VEGF. However, constructs of rFn in which the cell binding and VEGF-binding domains were physically linked dramatically enhanced these cellular responses, even more so than native Fn. rFn fragments containing only the cell-binding domain (FnIII<sub>9–10</sub>) did modestly enhance VEGF responses. These results may reflect the isolated contribution of the costimulatory signaling pathways that are known to exist between αβ and VEGFR-2 downstream to Erk. However, the current data suggest that these cooperative pathways are capable of a much more robust response when their receptors are occupied by a specialized VEGF/Fn complex. The rFnIII<sub>9–10/12–14</sub> construct...
dramatically increased early Erk phosphorylation in response to VEGF, as well as inducing a major late peak in Erk activation at 1.5 hour. Thus, the coordinated extracellular modulation of these ligand/receptor interactions imposes more stringent directions on the intracellular signaling responses to VEGF.

Several lines of evidence suggest that the binding interaction between VEGF and the Hep-II domain are complex. VEGF did not bind to any single isolated immobilized type III repeat, yet VEGF bound to the Fn moieties containing the type III12–14 repeats in continuity. This suggests that a more complex tertiary structure within the Hep-II domain is necessary for VEGF binding. We observed that the constructs rFnIII12–14 and rFnIII9–10/12–14 bound more VEGF than rFnIII12–14 alone. If FnIII9–10 itself has no intrinsic VEGF-binding properties (as we observed), why should the larger construct that includes FIII9–10 bind VEGF more avidly? These larger constructs (FnIII8–14 and FnIII9–10/12–14) likely assume a conformation that more optimally displays the VEGF-binding sites. This is supported by the surface plasmon resonance studies, which showed that rFnIII9–12–14 has a higher affinity for VEGF than native Fn. The bivalent Fn constructs encompassing both the integrin and VEGF-binding sites were also more biologically effective than native Fn. This again may be attributable to conformational differences, or their lack of large portions of the N and C-terminal regions of native Fn, which may play a negative modulatory role on VEGF function.

The heparin-binding properties of the FnIII12–14 domain are clearly important, as illustrated by the failure of the heparin
Figure 7. rFnIII9–10/12–14 promotes Erk and VEGFR-2 phosphorylation at low VEGF concentration. A and B, HUVECs were exposed to varying concentrations of VEGF in the absence (A) or presence (B) of rFnIII9–10/12–14 (0.2 μmol/L). Erk phosphorylation was determined by Western blotting. A representative blot from 2 separate experiments is shown. C, HUVEC cultures were exposed to when stimulated with VEGF. Our previous studies suggest that platelets may play a role in promoting angiogenesis. Recent studies by Kisucka et al have characterized the role platelets play in modulating angiogenesis in vivo.60 We also observed that blockade of α5β1 profoundly inhibits VEGF-mediated migration and differentiation of endothelial progenitor cells.22,23 These findings support the view espoused by Hynes and colleagues that α5β1 is a pivotal integrin for vascular development.6,37,38 The current experiments suggest that the mechanism of Fn-induced enhancement of VEGF activity arises from both the formation of an extracellular complex interacting with the cell surface receptors and the resulting promotion of costimulatory signaling from integrin and VEGF receptor. Simple engagement of integrin and growth factor receptors by free, independent ligands does not induce the same quality or quantity of signaling, especially the late sustained Erk phosphorylation. In support of our hypothesis, recent studies of a parallel system demonstrated that binding of basic fibroblast growth factor to fibronectin is required for the enhancement of basic fibroblast growth factor induced angiogenesis.39

More work will be needed to identify the interactive sites within VEGF and to define the role of the CS-1 domain of Fn also remains to be elucidated in this context. Further understanding of the structure/function relationship of these synergisms should permit the development and optimization of Fn-derived constructs that specifically amplify (or inhibit) the biological actions of VEGF.

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**Disclosures**

None.

**References**


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**mutant fragment to bind or modulate VEGF activity. An alternative explanation for the behavior of the heparin mutant protein might be that the mutations altered the conformation of the Fn fragment. Weighing against that possibility are the studies of Mostafavi-Pour, which suggest that these mutations do not seriously alter the conformation of the protein.26 Also, conformational changes of the mutant (or wild-type recombinant) protein would not explain why peptides selected from these heparin-binding domains successfully competed with rFnIII9–10/12–14 for binding VEGF.

However, the peptide competition experiments also suggest that heparin-binding is not the entire VEGF/Fn binding story. The heparin-binding site in III13 is thought to be the primary integrin binding site in Fn.33 Thus, this vWf heparin-binding peptide, a highly charged, classical heparin-binding domain, had relatively low affinity for VEGF. This same vWf protein domain has been shown to interfere with cell adhesive strength and chemokinesis, presumably because of its interference with cell syndecan binding to the Hep-II domain of Fn.33 Thus, this vWf heparin-binding domain may mimic the Fn Hep-II region, from the viewpoint of cell surface syndecans, but not from the perspective of VEGF. Finally, exogenous heparin was not required for rFn-VEGF binding (Figures 2 and 3). Heparin had the same mild stimulatory effect on the biological activity of rFn/VEGF complexes as its effect on VEGF alone (which is well described).

A number of mechanisms have been suggested for integrin/growth factor synergism, many emphasizing pathways for intracellular signaling crosstalk. Signaling from α5β1 ligation (and syndecan engagement) can support or reinforce the downstream signaling from VEGFR-2 to Erk.22 Conversely, VEGF-2 activation can lead to integrin activation.23 There exists some controversy as to whether the primary integrin synergizing with VEGF pathways is α5β1 or α5β3,24,34 This may be attributable to the type of ECM protein the cells are exposed to when stimulated with VEGF. Our previous studies suggest that VEGF/Fn complexes primarily cause a physical association between VEGFR-2 and α5β1, but not α5β3.22 Moreover, we have also shown that VEGF/Fn complexes, as well as hepatocyte growth factor/Fn complexes, exist and are released from thrombin stimulated degranulating platelets.22,35 These observations suggest that platelets may play a role in promoting angiogenesis. Recent studies by Kisucka et al have characterized the role platelets play in modulating angiogenesis in vivo.60 We also observed that blockade of α5β1 profoundly inhibits VEGF-mediated migration and differentiation of endothelial progenitor cells.22,23 These findings support the view espoused by Hynes and colleagues that α5β1 is a pivotal integrin for vascular development.6,37,38 The current experiments suggest that the mechanism of Fn-induced enhancement of VEGF activity arises from both the formation of an extracellular complex interacting with the cell surface receptors and the resulting promotion of costimulatory signaling from integrin and VEGF receptor. Simple engagement of integrin and growth factor receptors by free, independent ligands does not induce the same quality or quantity of signaling, especially the late sustained Erk phosphorylation. In support of our hypothesis, recent studies of a parallel system demonstrated that binding of basic fibroblast growth factor to fibronectin is required for the enhancement of basic fibroblast growth factor induced angiogenesis.39

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**rFn cDNA construction.** Individual modules of FnIII12, III13, III14 were amplified using primers containing BamHI and XhoI restriction sites. rFnIII12: 5’-GCTATTCTGCACCAACT-3’ and 5’-CTCCAGAGTGGTGAACAC-3’; rFnIII13; 5’-AATGTCAGGCCACCAAGA-3’ and 5’-AGTGGAGGCAGTGACGAC-3’; rFnIII14; 5’-GCCATTGATGCACCATCC-3’ and 5’-TGTCTTTTCTTCCAT-3’. To construct rFnIII8-11, rFnIII8-14 and rFnIII12-14 cDNA, amplification was carried out using primers containing SmaI and NotI restriction sites. Primers used were: 5’-GCTGTTCTTCCTCCACT-3’ and 5’-GGTTACTGCAGTCTGAAC-3’ for Fn(III)8-11; 5’-GCTATTCTGCACCAACT-3’ and 5’-TGTCTTTTCTTCCAT-3’ for FnIII12-14; 5’-GCTGTTCTTCCTCCACT-3 and 5’-TGTCTTTTCTTCCAT-3’ for FnIII8-14. All PCR products were digested with the appropriate restriction enzyme and cloned into pGEX-5X2 vector except for rFnIII8-14 which was cloned into pMT/Bip-His vector. The rFnIII9-10/12-14 DNA was created by sequential PCR. In separate reactions, rFnIII9-10 and rFnIII12-14 were amplified by primers containing complementary 5’ extensions. The PCR products were mixed and annealed followed by extension using KOD DNA polymerase. The combined rFnIII9-10/12-14 DNA was then amplified using the forward III9 primer and the reverse III14 primer. All cloned Fn fragments were sequenced to verify no base changes.

**Expression and purification of recombinant Fn fragments.** The rFnIII8-14 fragment was expressed as a 6X histidine tagged fusion protein in Drosophila S2 cells. All other Fn fragments were expressed in bacteria (BL21) as glutathione-S-transferase (Gst) fusions. For Drosophila S2 cell protein production, the pMT/Bip-FnIII8-14 vector was transfected into S2 cells using calcium
phosphate and grown in complete Schneider’s insect medium. Stable cell lines were selected using 35 µg/ml Blastacidin. To produce proteins, S2 cells were grown in SFM 900 serum-free medium and induced with 0.75 mmol/L CuSO₄ for 4 days. Conditioned medium was incubated with S-sepharose beads overnight at 4°C and proteins were eluted by FPLC over a 0.1-1 mol/L salt gradient in 20 mM sodium phosphate, pH 7.5. The eluted rFnIII₈-₁₄ was further purified using Talon beads and bound rFnIII₈-₁₄ was cleaved from the beads with factor Xa. For proteins expressed in bacteria, lysates were incubated with glutathione-sepharose beads. After washing with PBS, fragments were released from the beads by cleaving with factor Xa. After removing factor Xa (Xarrest agarose; Novagen), the purified proteins were dialyzed against PBS and their purity was assessed by Bis-Tris PAGE. Fn fragments used in HUVEC migration, proliferation and cell signaling assays were treated with Acticlean Etox beads (Sterogene) to remove endotoxin. Levels of endotoxin were less than 0.1U per µg protein as determined by LAL assay.
Online Table I. Analysis of the kinetics of VEGF$_{165}$ binding to native Fn and rFnIII$_{9-10/12-14}$.

<table>
<thead>
<tr>
<th>Immobilized</th>
<th>Analyte</th>
<th>$K_D$ (nM)</th>
<th>$k_{on}$ (M$^{-1}$·sec$^{-1}$)</th>
<th>$k_{off}$ (sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>native Fn</td>
<td>VEGF$_{165}$</td>
<td>129.3</td>
<td>2.41x10$^4$</td>
<td>3.12x10$^{-3}$</td>
</tr>
<tr>
<td>rFnIII$_{9-10/12-14}$</td>
<td>VEGF$_{165}$</td>
<td>73.5</td>
<td>3.45x10$^4$</td>
<td>2.53x10$^{-3}$</td>
</tr>
</tbody>
</table>