研究成果の刊行に関する一覧表

書籍

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研究成果の刊行物・別刷

SUGAR-IMMOBILIZED GOLD NANO-PARTICLES (SGNP): NOVEL BIOPROBE FOR THE ON-SITE ANALYSIS OF THE OLIGOSACCHARIDE-PROTEIN INTERACTIONS

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Introduction

Oligosaccharides are increasingly being recognized as important partners in receptor-ligand binding and cellular signaling[1]. Specific structural attributes of the oligosaccharide determine their biological functions, through distinct binding interactions with proteins, other oligosaccharides, or cells. We have developed a chip technology, in which structurally defined oligosaccharides are two-dimensionally immobilized on a surface, which mimics nature[2]. The combination of our technology with Surface Plasmon Resonance (SPR) is a very powerful tool for the real-time study of the specific interactions between biological molecules, and could be used as a high-throughput screening method or as a novel diagnosis technology, since the experiment can be done without any labeling of targets. However, SPR experiments need particular and expensive equipment, and so can hardly be performed on-site, such as a patient's bedside in the hospital. To establish an on-site analysis, we apply our immobilization method to gold nano-particles (GNP) to prepare sugar-immobilized gold nano-particles (SGNP), and report the visual detection of the sugar-protein interaction and the isolation of the specific sugar-binding protein in this manuscript.

Experimental

Materials. All reagents were used without further purification. Sodium tetrachloroaurate was purchased from Nacalai Tesque (Kyoto, Japan). Proteins were purchased as follows: Concanavalin A (Con A) from EY-Laboratories (CA, USA); RCA120 from HONEN (Tokyo, Japan); bovine Serum Albumin (BSA) from (MS, USA). Reagents for SDS-PAGE were obtained from Nacalai Tesque.

Instrumentation. UV-VIS spectra were measured using Nano-drop (Nano drop Tech., DW, USA). Mass spectrometry was performed by Voyager DE-Pro or Mariner™ (AppliedBiosystems, CA, USA). JEM-3010 (JEOL, Tokyo, Japan) was used as a transmittance electron microscope (TEM) for GNP and SGNP. NMR spectra of the compounds were obtained on a JEOL ECA-600 spectrometer.

Synthesis of Glcα4Glc-mono (Ligand-conjugate containing α-D-glucoside). Mono-valent linker compound (10.0 mg, 34 μmol) was dissolved in 1.0 ml of dimethylacetoamide (DMAc). To the solution, were added maltose (12.2 mg, 34 μmol) dissolved in 0.8 ml of distilled water and 2 ml of acetic acid. The reaction solution was kept on stand at 37 °C for 24 h. To the solution, $NaCNBH_3$ (21.3 mg, 340 μmol) dissolved in 2 ml of distilled water was added. Then, the reaction solution was further kept on stand at 37 °C. After 72 h, the reaction was quenched by adding 2 ml of acetone. After removing organic solvent by evaporation, the reaction mixture was lyophilized. The purification was done by ODS and GPC. The purified product was characterized by ^1H NMR and MS.

Synthesis of Galβ4Glc-mono (Ligand-conjugate containing β-D-galactoside). By a similar protocol described above, the objected compound was prepared. Lactose was used instead of maltose.

Synthesis of Glcα4Glc-GNP (α-D-Glucoside immobilized SGNP). To 1 mM of aqueous solution of $NaAuCl_4$, $NaBH_4$ was added to 5 mM (final concentration) with stirring. Then Glcα4Glc-mono, a ligand-conjugate, was added with stirring. Then, the reaction solution was dialyzed against distilled water and phosphate-baffured saline containing 0.05% tween-40 (PBS-T).

Synthesis of Galβ4Glc-GNP (β-D-Galactose immobilized SGNP). A similar protocol described above was applied to prepare Galβ4Glc-GNP. The ligand-conjugate, Galβ4Glc-mono, was used instead of Glcα4Glc-mono.

Binding experiment. 50 μL of SGNP colloid solution (OD at 520 nm = 0.3 /mm) was placed in 96 well plastic micro plate. To each well, proteins dissolved in PBS-T (50 μL) were added with changing the concentration. The plate was gently rotated at 50 rpm for 1 h.

Isolation of protein. 50 μL of SGNP (OD at 520 nm = 0.3 /mm, PBS-T) was placed in 1.5 mL eppendorf tube. To the tube, protein solution was added. The tube was rotated at 50 rpm for 2 h to obtain the precipitate of SGNP. After removing the supernatant, the precipitate was washed with PBS-T and distilled water. Then, aqueous solution of monosaccharide (ex. D-glucose in the case of Glcα4Glc-GNP; D-galactose in the case of Galβ4Glc-GNP) was added to the tube. After dissolving the precipitate into gold colloidal solution using vortex mixer for 10 seconds, 10 μL of the colloidal solution was removed for the detection of the protein by SDS-PAGE.

Results and Discussion

Preparation of ligand-conjugates. The synthesis of Glcα4Glc-mono was depicted in **Figure 1.** An optimized reductive amination reaction and well-designed linker compound[2], in which aromatic amino group and the cyclic disulfide moiety were key units, were selected. The yield was about 50%, but major loss was occurred at the purification stage.

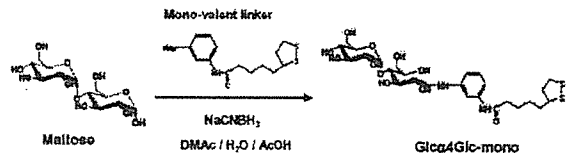

Figure 1. Preparation of ligand-conjugate (Glcα4Glc-mono)

Preparation of sugar-immobilized gold nano-particle (SGNP). GNP was first prepared according to Brust *et al.*[3] using $NaBH_4$ as a reducing reagent. Without isolation of GNP, the ligand-conjugate was added into the preparing solution of GNP *in situ*. The concentration of the ligand-conjugate affected the preparation of SGNP. As shown in **Figure 2**, immature SGNP was aggregated during the dialysis. The best result was obtained when 100 μM (final concentration) of ligand conjugate was used against 1 mM of sodium tetrachloroaurate for the starting GNP solution. The color of SGNP was purple, showing λ_{max} at 520 nm.. The size of SGNP measured by TEM (**Figure 3**) were about 2 - 10 nm, which were smaller than GNP (10 - 40 nm) prepared by the conventional method using citric acid. From the size and the optimized concentration of ligand-conjugate, it was speculated that about 8 ligand-conjugates were immobilized on one molecule of GNP in average. The obtained SGNP is very stable and can be stored as lyophilized powder.

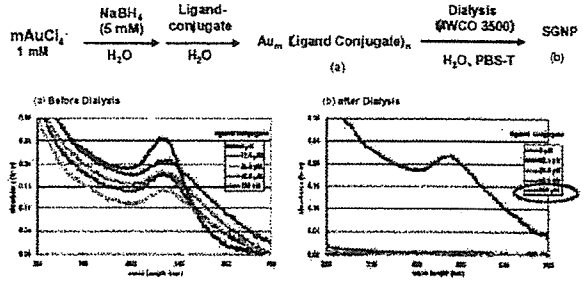

Figure 2. Protocol for the preparation of SGNP (upper) and changes of UV-VIS spectra during the preparation (lower).

Binding experiment using SGNP. Figure 4 shows the results of the interaction between protein and sugar moiety immobilized on the GNP. In the case of Con A, which is known to have a binding potency with α-glucose or α-mannose, the aggregates were formed in only Glcα4Glc-GNP series, not in Galβ4Glc-GNP. In contrast, in the case of RCA120, which is a α/β-galactose binding lectin protein, the aggregates were formed in Galβ4Glc-GNP, but not in Glcα4Glc-GNP. In the case of BSA, which is not a lectin protein used as negative control, no aggregates were formed in both cases. It is important to note that those changes can be detected visually. These results indicate that

our SGNP may have good property for the binding interaction between oligosaccharides and proteins on-site. The optical density (OD) at 520 nm of supernatant (solution) was monitored and plotted against the concentration of protein in **Figure 4**. It was cleared that the specific binding between α-Glc and Con A or β-Gal and RCA120 was detected. Also from this OD-values, the dissociation constant (K_D) was calculated: 490 nM for ConA and 330 nM for RCA120. These values are similar to those obtained by SPR experiments.

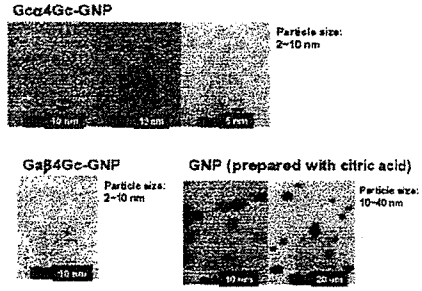

Figure 3. TEM images of SGNP (Glcα4Glc-GNP: upper, Galβ4Glc-GNP: lower left) and GNP prepared by the conventional method using citric acid. Bars in the images represent scales.

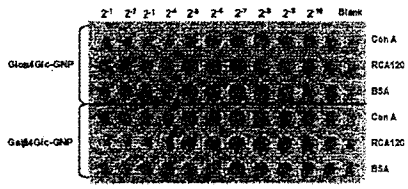

Figure 4. Results of the binding interaction between proteins and sugar moiety immobilized on SGNP. Protein solutions were diluted to 1/2 from the original concentrations.

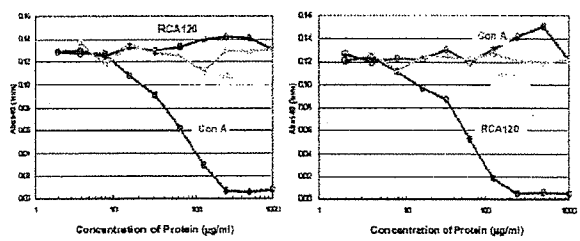

Figure 5. Results of the binding interaction between proteins and sugar moiety immobilized on SGNP monitored by OD at 540 nm. Left: Glcα4Glc-GNP; Right: Galβ4Glc-GNP.

Isolation and purification of proteins bound to SGNP. SGNPs were applied for an easy and quick isolation of carbohydrate binding lectin protein as shown in **Figure 6**. SGNP was aggregated by the addition of the appropriate protein (Con A to Glcα4Glc-GNP, RCA 120 to Galβ4Glc-GNP). After washing, when appropriate monosaccharide (D-glucose to Glcα4Glc-GNP, D-galactose to Galβ4Glc-GNP) was added, the aggregate turned into a colloidal solution. The effect of the concentration is shown in **Figure 7**. It was found the OD at 530 nm was recovered, suggesting a re-formation of SGNP colloidal solution. The bound protein in the aggregates was eluted as shown in **Figure 8** by the competitive binding of the monosaccharide to the protein bound to immobilized sugar moiety on GNP, suggesting quick isolation of the target protein. A similar protocol was applied for the easy purification of protein from the mixture. **Figure 9** shows the results of SDS-PAGE. From the mixture of Con A and BSA, Con A was purified by one step using Glcα4Glc-GNP.

Conclusions

It was obvious that sugar-immobilized gold nano-particle was very useful for the on-site detection of binding interaction between sugar-moiety and protein, and for the quick and easy isolation/purification of carbohydrate binding proteins. We have prepared so far more than 30 kinds of SGNP. More applications of SGNP, such as diagnosis method for specific diseases upon infection, are now on-going.

Acknowledgements. The authors of this paper would like to thank SHORAI Foundation for Science and Technology, SUZUKEN Memorial Foundation, Japan Science and Technology Agency (JST), and Japan Ministry of Health, Labor and Welfare for financial support of this research.

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(2) Suda, Y. et al., WO 2004/022583 A1; WO 2004/022565 A1.
(3) Brust M., *et. al, J. Chem. Soc. Chem., Commun.*, **1994**, 801.

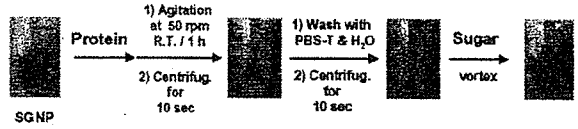

Figure 6. Protocol for the isolation/purification of protein(s) which bound immobilized sugar moiety specifically.

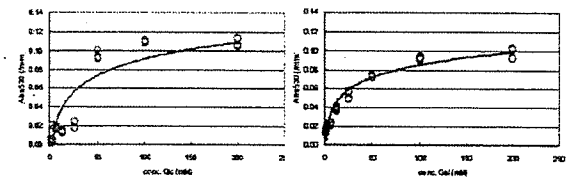

Figure 7. Recovery of the colloidal solution of SGNP by the addition of monosaccharide. Left: D-glucose for Glcα4Glc-GNP, Right: D-galactose for Galβ4Glc-GNP.

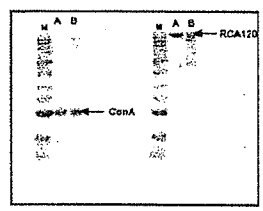

Figure 8. SDS-PAGE of colloidal solution of SGNP. Left: Con A was added to Glcα4Glc-GNP, Right: RCA 120 was added to Galβ4Glc-GNP. M: marker protein, A: original protein solution, B: colloidal solution after the addition of monosaccharide.

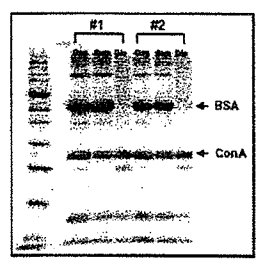

Figure 9. SDS-PAGE of colloidal solution of SGNP. #1: 500 μg/ml of BSA and 300 μg/ml of Con A was mixed in the original solution (Org). #2: 250 μg/ml of BSA and 300 μg/ml of Con A was mixed in the original solution (**Org**). **Sup** represents supernatant of the aggregate. **Dis** represents the recovered colloidal solution.

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

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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 $FnIII_{13-14}$ abolished VEGF binding, and peptides corresponding to the heparin-binding sequences in $FnIII_{13-14}$ inhibited VEGF binding to Fn. Fn fragments containing both the $\alpha_5\beta_1$ 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 $\alpha_5\beta_1$ 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 $\alpha_5\beta_1$,[17] and by Moro et al for epidermal growth factor receptor.[18] Others have shown that β_1 activation by Fn can modulate VEGF responses,[19] and that β_3 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 $\alpha_v\beta_3$ and $\alpha_5\beta_1$ 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, $\alpha_5\beta_1$ and VEGFR-2.[22,23] We found VEGF bound to Fn and that platelets secreted VEGF/Fn complexes. In the presence of the

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ligands VEGF and Fn, their corresponding receptors coimmunoprecipitated. 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 $\alpha_5\beta_1$ 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 obtained by PCR amplification using Fn cDNA as template. We used the following nomenclature for describing the recombinant Fn constructs: $rFnIII_{9-10}$ indicates recombinant Fn protein encompassing type III repeats 9 to 10; $rFnIII_{9-10/12-14}$, a single protein encompassing type III repeats 9 to 14, omitting 11. A $rFnIII_{12-14}$ 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 (Neuro Probe 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, 5×10^5 HUVECs were plated onto 60-cm^2 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 (2×10^6 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 $rFNIII_{9-10/12-14}$ 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.

^{125}I-VEGF Solid-Phase Binding Assays

The measurement of direct binding of ^{125}I-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 $III_{9-10/12-14}$ or $rFnIII_{12-14}$ immobilized on the plates.

Surface Plasmon Resonance Analysis

Surface plasmon resonance analysis (SPR) binding experiments were conducted to measure the equilibrium binding parameters between $VEGF_{165}$ and native fibronectin or $rFnIII_{9-10/12-14}$. 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*-hydroxysuccinimidyl coupling, sensograms recorded over a range of VEGF concentrations (25 to 800 nmol/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 ^{125}I-$VEGF_{165}$ binding to different immobilized recombinant Fn fragments. These experiments were all conducted in the absence of exogenous heparin, indicating that heparin is not a

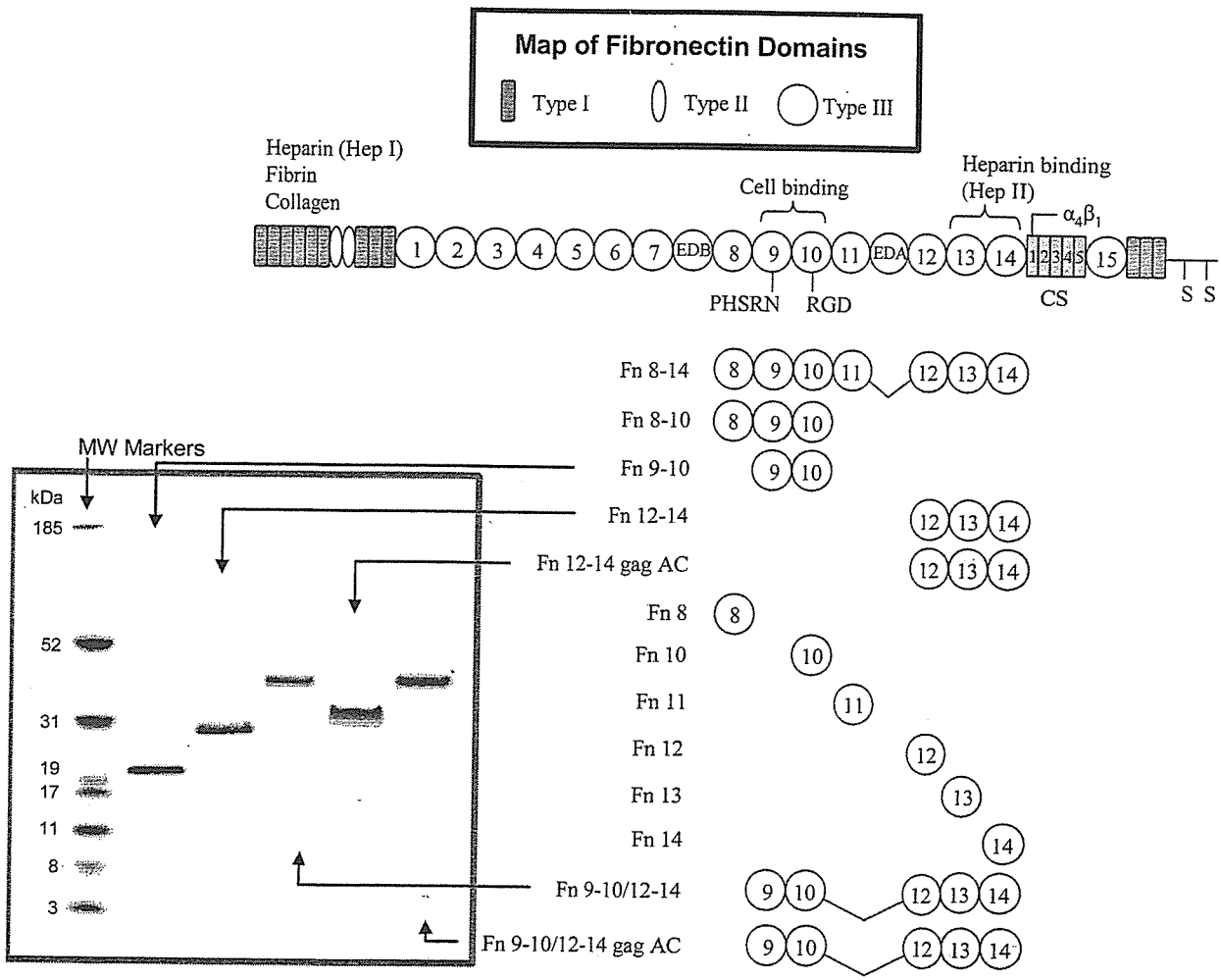

Figure 1. Schematic diagram of the domain structure of human Fn showing type I, type II, type III and their functions. Also diagramed are the recombinant Fn type III domains used in this study, and on the left is a representative SDS-PAGE gel of purified fragments.

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 $\alpha_5\beta_1$ 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

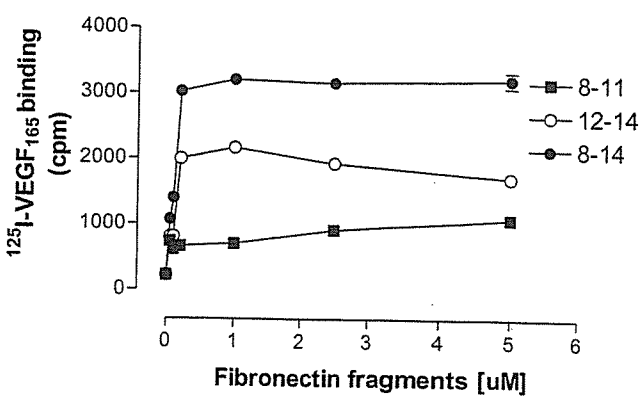

Figure 2. Concentration dependent ^{125}I-VEGF binding to immobilized rFn fragments. ^{125}I-VEGF was incubated for 2 hours on wells coated with increasing concentrations of rFn fragments. After washing, bound VEGF was eluted with 0.1 mol/L NaOH/1% SDS and radioactivity quantified in a gamma counter. Data are presented as the mean of triplicate determinations±SEM.

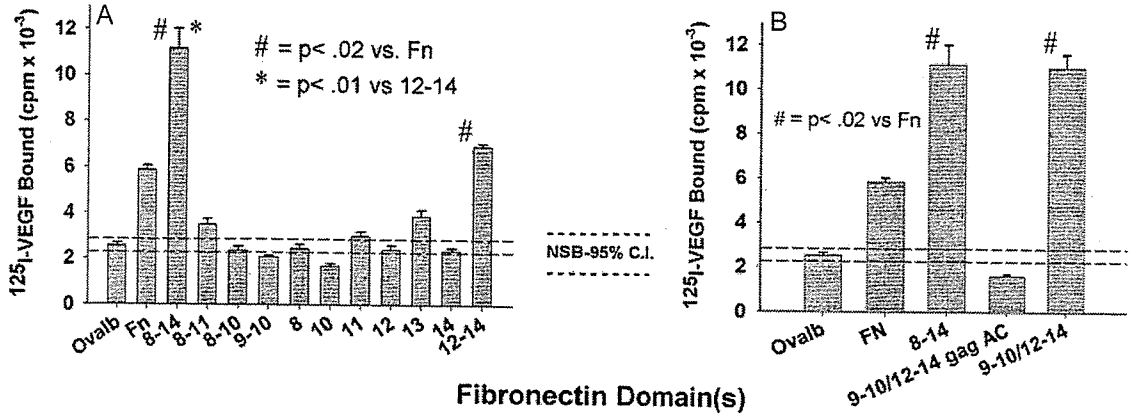

Figure 3. Mapping the VEGF-binding site on C-terminal Fn. A and B, ^{125}I-VEGF was incubated for 2 hours on wells coated with either individual or continuous rFn type III domains (0.2 μmol/L) (A) or wells coated with rFn molecules encompassing both the integrin (9 to 10) and heparin-binding domains (12 to 14) (B). GagAC denotes mutation of the heparin-binding domains on type III module 13 and 14. After washing, bound VEGF was eluted with 0.1 mol/L NaOH/1% SDS and radioactivity quantified in a gamma counter. Data are the means±SEM of 3 to 4 independent experiments performed in triplicate. Dotted lines are 95% confidence intervals for control binding to albumin.

VEGF. To that end, the construct $rFnIII_{9-10/12-14gagAC}$ 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.

Relative Inhibition of ^{125}I-$VEGF_{165}$ Binding by Heparin Binding Peptides

Protein	Peptide Sequence	IC50 (μmol/L)
$rFnIII_{13}$	1814 vspprrarvtdatettitiswrtktetitgfq	43
$rFnIII_{13}$ (truncated)	1814 pprrarv	>1000
$rFnIII_{14}$	1926 praritgyiikyekpgspprevvprprpgv	0.4
$rFnIII_{14}$	1971 iyvialknnqkspligrkkt	150
von Willebrand factor	568 kdrkrselrriasqvk	250

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 $\alpha_5\beta_1$ 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 $rFnIII_{12-14}$ alone. Most of the proteins containing an integrin-binding domain (eg, vitronectin, rFn_{9-10}, and $rFn_{9-10/12-14gagAC}$) supported some migration to VEGF. In contrast, all bivalent matrices encompassing both the $\alpha_5\beta_1$ 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 $rFnIII_{12-14}$. 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 $rFnIII_{12-14}$). Addition of heparin (1 μg/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

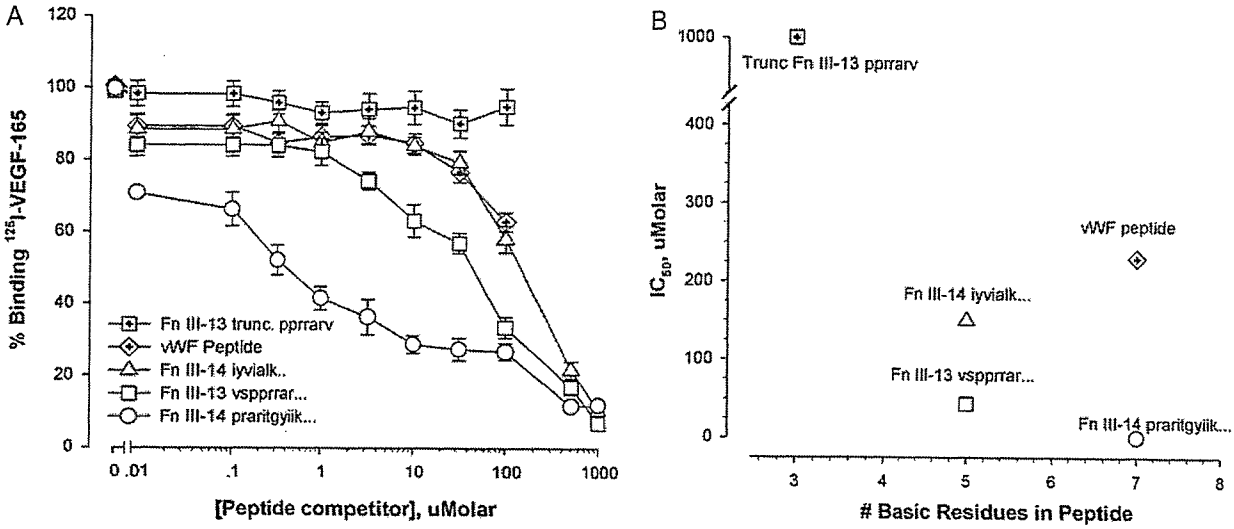

Figure 4. Peptide inhibition of VEGF binding to $rFnIII_{9-10/12-14}$. ^{125}I-VEGF/Fn binding assays were performed in the presence of the peptides listed in the Table. A, Results are expressed as the percentage of VEGF binding observed in the absence of competitor (100%) and are the means±SEM of 4 to 6 independent experiments performed in triplicate. B, IC_{50} (μmol/L) was interpolated for each peptide and plotted against the total number of basic residues/peptide.

(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,[22] 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 $rFnIII_{9-10/12-14}$ induced an even higher early peak and a second peak of Erk activation at approximately 1.5 hour. A mixture of $rFnIII_{9-10}$ and $rFnIII_{12-14}$ (at the same individual concentrations as other fragments tested) did not induce any late Erk activation. When tested individually, the cell binding ($rFnIII_{9-10}$) or VEGF-binding domains ($rFnIII_{12-14}$) did not induce any late Erk activation. The divalent recombinant Fn fragment $rFnIII_{9-10/12-14}$, significantly enhanced VEGF-induced Erk phosphorylation even at very low VEGF concentrations, between 0.1 to 0.5 ng/mL (Figure 7A and 7B). We next examined the influence of $rFnIII_{9-10/12-14}$ on the phosphorylation status of VEGFR-2 activated by a low dose of VEGF. Figure 7C shows that VEGF alone (1 ng/mL) did not significantly induce VEGFR-2 phosphorylation. However, in the presence of $rFnIII_{9-10/12-14}$, significant VEGFR-2 phosphorylation was observed. Stimulation of cells with VEGF and either $rFnIII_{9-10}$ or $rFnIII_{12-14}$ did not show enhanced VEGFR-2 phosphorylation.

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.[24,25,30] The Hep-II site is a major syndecan-binding site that plays a role in focal adhesion and stress fiber formation.[32] 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 $FnIII_{14}$ 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 ($FnIII_{9-10}$) did modestly enhance VEGF biological responses. These results may reflect the isolated contribution of the costimulatory signaling pathways that are known to exist between $\alpha_5\beta_1$ 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 $rFnIII_{9-10/12-14}$ construct

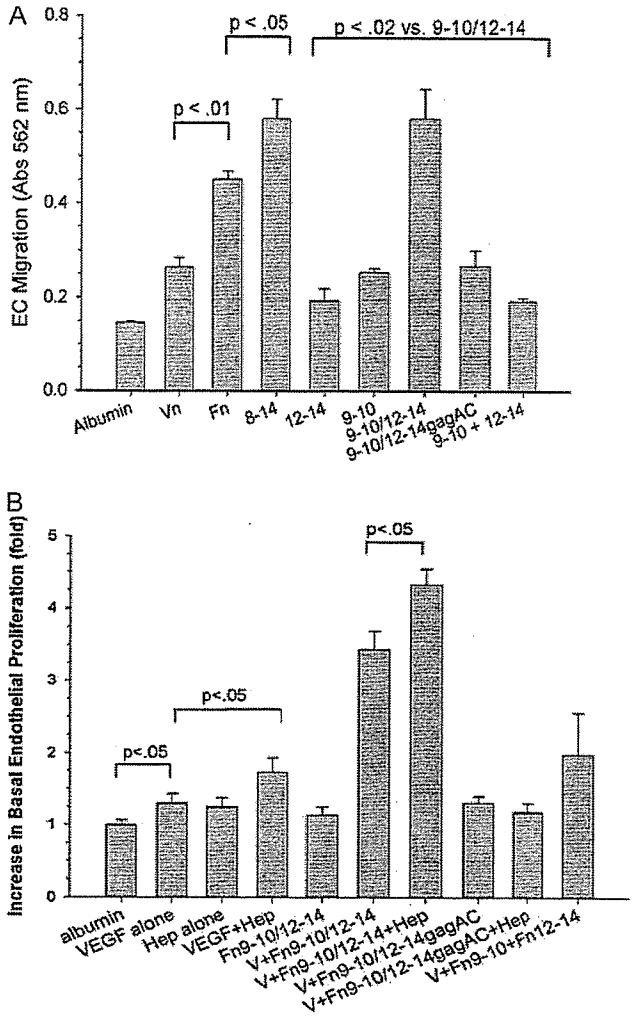

Figure 5. A, EC migration to VEGF in the presence of rFn fragments. HUVECs suspended in serum-free MCDB-131 medium containing 0.1% ovalbumin were seeded on to ChemoTx microtiter plates and migration assay performed as described in Materials and Methods. Experiments were performed in triplicate and results expressed as mean±SEM. The final bar (indicated by 9-10 + 12- 14) signifies treatment with a mixture of separate fragments of $rFnIII_{9-10}$ and $rFnIII_{12-14}$, each at 0.2 μmol/L. B, EC proliferation in response to VEGF and rFn fragments. HUVECs (3000 cells/well) in 96-well plates were incubated in MCDB131 medium containing Fn-depleted FBS (0.25%). Cells were incubated with VEGF alone (10 ng/mL), VEGF (V) with native Fn (5 ng/mL), or rFn fragments (0.2 μmol/L). In some experiments, heparin (hep) was added at a final concentration of 1 μg/mL. Cell growth was determined after 72 hours. Experiments were done in triplicate and results are expressed as mean±SEM.

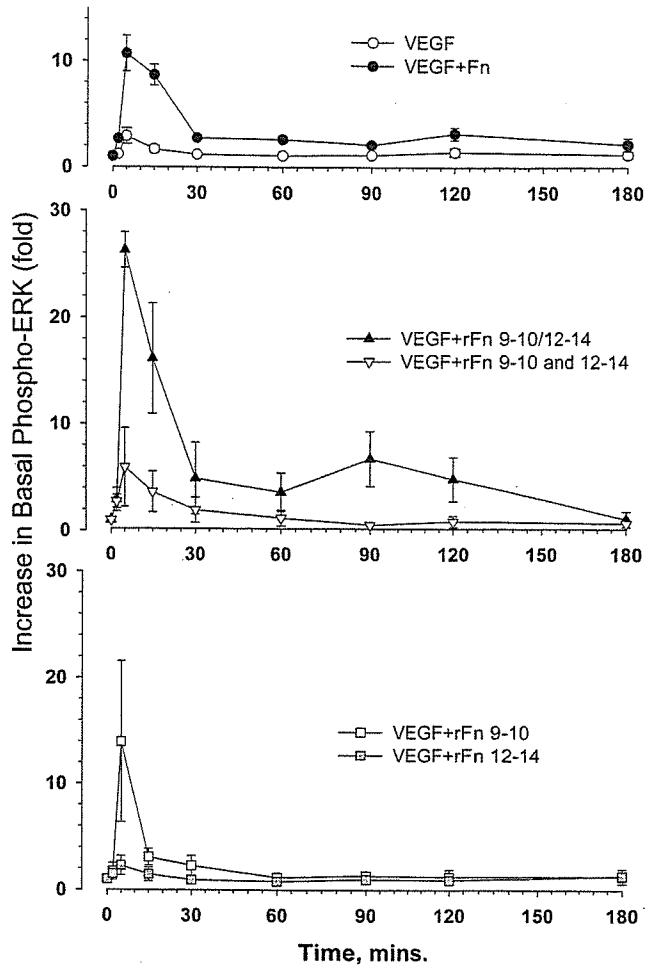

Figure 6. HUVEC Erk phosphorylation in response to VEGF and Fn moieties. HUVECs were exposed to 10 ng/mL of $VEGF_{165}$, combined with the indicated Fn proteins (0.2 μmol/L). At the time points, cells were harvested and processed to measure phospho-Erk and total Erk protein by Western blotting. Each point on the graph is the mean of data obtained from 4 to 5 separate experiments and quantified as described in Materials and Methods.

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 III_{12-14} 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 $rFnIII_{8-14}$ and $rFnIII_{9-10/12-14}$ bound more VEGF than $rFnIII_{12-14}$ alone. If $FnIII_{9-10}$ itself has no intrinsic VEGF-binding properties (as we observed), why should the larger construct that includes $FIII_{9-10}$ bind VEGF more avidly? These larger constructs ($FnIII_{8-14}$ and $FnIII_{9-10/12-14}$) likely assume a conformation that more optimally displays the VEGF-binding sites. This is supported by the surface plasmon resonance studies, which showed that $rFnIII_{9-10/12-14}$ 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 $FnIII_{12-14}$ domain are clearly important, as illustrated by the failure of the heparin

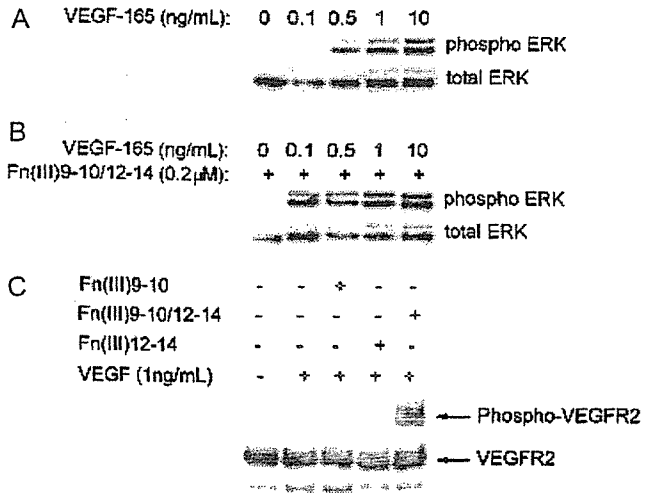

Figure 7. rFnIII$_{9-10/12-14}$ promotes Erk and VEGFR-2 phosphorylation at low VEGF concentration. A and B, HUVECs were exposed to varying concentrations of VEGF in the absence (A) or presence (B) of rFnIII$_{9-10/12-14}$ (0.2 μmol/L). Erk phosphorylation was determined by Western blotting. A representative blot from 2 separate experiments is shown. C, HUVEC cultures were stimulated with a low dose of VEGF (1 ng/mL) in the absence or presence of recombinant Fn fragments (0.2 μmol/L). Cell lysates were immunoprecipitated with anti–VEGFR-2 rabbit monoclonal antibodies followed by Western blotting with anti-phosphoVEGFR-2 rabbit monoclonal antibodies. Blots were stripped and reprobed with anti–VEGFR-2 antibodies. Experiments were repeated twice with similar results.

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 rFnIII$_{9-10/12-14}$ 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 III$_{13}$ is thought to be the dominant interactive site for heparins,[24,25] yet its peptide domain was not the most potent at blocking VEGF binding, nor did its truncated form (the core heparin-binding residues) retain activity. Likewise, comparisons of peptide charge versus inhibitory potency suggest that nonelectrostatic, protein/protein interactions also account for VEGF/Fn binding. The 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 $\alpha_5\beta_1$ ligation (and syndecan engagement) can support or reinforce the downstream signaling from VEGFR-2 to Erk.[22] Conversely, VEGFR-2 activation can lead to integrin activation.[21] There exists some controversy as to whether the primary integrin synergizing with VEGF pathways is $\alpha_5\beta_1$ or $\alpha_v\beta_3$.[21,34] 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 $\alpha_5\beta_1$, but not $\alpha_v\beta_3$.[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.[36] We also observed that blockade of $\alpha_5\beta_1$ profoundly inhibits VEGF-mediated migration and differentiation of endothelial progenitor cells.[22,23] These findings support the view espoused by Hynes and colleagues that $\alpha_5\beta_1$ 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 fibrinogen 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.

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Immobilization and Clustering of Structurally Defined Oligosaccharides for Sugar Chips: An Improved Method for Surface Plasmon Resonance Analysis of Protein–Carbohydrate Interactions

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Oligosaccharides are increasingly being recognized as important partners in receptor–ligand binding and cellular signaling. Surface plasmon resonance (SPR) is a very powerful tool for the real-time study of the specific interactions between biological molecules. We report here an advanced method for the immobilization of oligosaccharides in clustered structures for SPR and their application to the analysis of heparin–protein interactions. Reductive amination reactions and linker molecules were designed and optimized. Using mono-, tri-, or tetravalent linker compounds, we incorporated synthetic structurally defined disaccharide units of heparin and immobilized them as ligands for SPR. Their binding to an important hemostatic protein, von Willebrand factor (vWf), and its known heparin-binding domain was quantitatively analyzed. These multivalent ligand conjugates exhibited reproducible binding behavior, with consistency of the surface conditions of the SPR chip. This novel technique for oligosaccharide immobilization in SPR studies is accurate, specific, and easily applicable to both synthetic and naturally derived oligosaccharides.

INTRODUCTION

Oligosaccharides of two to ten sugar moieties are responsible for many important biological functions (*1*). Both the specific structural attributes of the minimum oligosaccharide unit and the spacing and distribution of these key binding units determine the ultimate biological function of heparins and heparin-like glycosaminoglycans (*2*). Often, the minimal oligosaccharide unit with a structurally specific interaction may be as small as a di- or trisaccharide (*3*, *4*). However, for full biological activity, these key binding units must often be presented in repeating units or clusters, appropriately spaced to interact with the heparin-binding sites of the cognate protein. Two major problems arise in the study of these structure–function relations. First, it is not easy to obtain large quantities of a homogeneous, structurally defined oligosaccharide. They cannot be expressed recombinantly, like proteins, and therefore, long and tedious chemical processes are necessary to synthesize or derive them from natural sources. The second problem is that an individual oligosaccharide motif may have low affinity but in biological systems they are often presented in clustered or repeating forms, which contribute significantly to their potency and affinity. We hypothesized that a chip or an array technology, in which structurally defined key oligosaccharide binding units are two-dimensionally immobilized on a surface, would be an effective method to solve these problems and could be used as a high-throughput screening method.

A number of investigators have tackled the challenges of oligosaccharide immobilization. Techniques for self-assembled monolayers have been described by Horan et al. (*5*), Mrksich and co-workers (*6*, *7*), and others. However, no method has so far been devised that both is procedurally simple and does not significantly modify or potentially interfere with the biological activity of the oligosaccharide. Most of these techniques require significant chemical modifications of the glycans, potentially obscuring the interpretation of structure–function relations. For example, conventional methods have involved chemical modifications at the 1-position of the carbohydrate, such as amination or allylation (*8*, *9*). But these approaches invariably put functional sulfate or phosphate groups at risk. Finally, currently established methods do not afford the opportunity to control the clustering or multimerization of oligosaccharide functional domains. To this end, we pursued novel immobilization strategies for oligosaccharides, which would be technically simpler and less destructive, making them useful to a wider group of investigators. For detection, we chose surface plasmon resonance (SPR) technology, which permits the real-time analysis of molecular binding using microgram quantities of analyzing materials, without further labeling (*10*–*14*). It can measure the binding affinity and on (association) and off (dissociation) rates and is useful for high-throughput screening of new drug targets.

In previous work, we had shown that a specific disaccharide unit in heparin, *O*-(2-deoxy-2-sulfamido-6-*O*-sulfo-α-D-glucopyranosyl)-(1→4)-2-*O*-sulfo-α-L-idopyranosyluronic acid (abbreviated as GlcNS6S-IdoA2S), was a key unit responsible for heparin binding to human platelets (*15*) and von Willebrand factor (vWf) (*16*). We also found that the clustering of these disaccharides significantly enhanced the interaction (*17*, *18*). To systematically investigate heparin's binding interactions, we

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