

A Catalytic Role of Heparin within the Extracellular Matrix*

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We investigated the mechanism by which heparin enhances the binding of vascular endothelial growth factor (VEGF) to the extracellular matrix protein fibronectin. In contrast to other systems, where heparin acts as a protein scaffold, we found that heparin functions catalytically to modulate VEGF binding site availability on fibronectin. By measuring the binding of VEGF and heparin to surface-immobilized fibronectin, we show that substoichiometric amounts of heparin exposed cryptic VEGF binding sites within fibronectin that remain available after heparin removal. Measurement of association and dissociation kinetics for heparin binding to fibronectin indicated that the interaction is rapid and transient. We localized the heparin-responsive element to the C-terminal 40-kDa Hep2 domain of fibronectin. A mathematical model of this catalytic process was constructed that supports a mechanism whereby the heparin-induced conformational change in fibronectin is accompanied by release of heparin. Experiments with endothelial extracellular matrix suggest that this process may also occur within biological matrices. These results indicate a novel mechanism whereby heparin catalyzes the conversion of fibronectin to an open conformation by transiently interacting with fibronectin and progressively hopping from molecule to molecule. Catalytic activation of the extracellular matrix might be an important mechanism for heparin to regulate function during normal and disease states.

Heparin and heparan sulfate are linear polysaccharides composed of repeating glucosamine-hexuronic acid disaccharide units (1). Heparan sulfate chains are found attached to core proteins in proteoglycans on cell surfaces and within the extracellular matrix of nearly all mammalian cells and tissues (2). In contrast, heparin is stored exclusively in the granules of certain subsets of mast cells (3). The physiological role of heparin has not been fully explored. It is known that heparin is important for the storage of the numerous proteases in the mast cell granules (4–6). Several studies have suggested a correlation between stimulated, degranulating mast cells and growth and

metastasis of several types of tumors (7, 8). Mast cells contain numerous mediators that are likely to affect several pathways of tumor progression, including immunosuppression, enhancement of angiogenesis, degradation of the extracellular matrix, and stimulation of tumor cell mitosis (7, 9). However, the role that heparin release upon mast cell degranulation plays in these pathways is unclear. Some studies suggest that mast cell heparin promotes mitosis and migration of endothelial cells (10). Moreover, the anticoagulant properties of heparin prevent the formation of thrombi in the newly formed vessels (11). Other studies show that heparin has the ability to inhibit tumor growth and metastasis (12–15), and there is some evidence that it may do so through an indirect mechanism, by interfering with the interaction between fibroblasts and the adjacent tumor cells (16).

Although heparin and heparan sulfate share similar chemical backbones, heparin exhibits a higher degree of sulfation and a more uniform distribution of sulfate groups along the chain, and it contains almost exclusively the iduronic epimer of the hexuronic acid residue (17). These properties are likely to determine the specificity of interactions between heparin and heparan sulfate with proteins (18).

Fibronectin, a major component of extracellular matrices, is a glycoprotein consisting of several individually folded domains connected with flexible linker regions (19). Interdomain interactions are important determinants of the overall tertiary structure of the protein (20–22). Fibronectin is able to interact with heparin and heparan sulfate, and there is evidence that this interaction affects the structure of the protein (23–28). In a previous study, we demonstrated that heparin mediates a conformational change in fibronectin from a compact to a more extended conformation, potentially by interfering with interdomain interactions (29). One consequence of the conformational change is the exposure of vascular endothelial growth factor (VEGF)² binding sites on the surface of fibronectin, leading to increased VEGF binding. Fibronectin has been implicated as a key mediator of a number of processes within vascular biology (30–32) and particularly in VEGF signaling (33–39).

In the present study, we investigated the mechanism by which heparin alters fibronectin conformation, leading to increased VEGF binding. We found that substoichiometric amounts of heparin are able to mediate the conformational change, as monitored by the increased capacity of fibronectin to bind VEGF, without the formation of a stable complex between

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² The abbreviations used are: VEGF, vascular endothelial growth factor; PBS, phosphate-buffered saline; ECM, extracellular matrix.

fibronectin, heparin, and VEGF. We propose that this mechanism reflects a new catalytic biological function of heparin, whereby the structure of the extracellular matrix (ECM) is modulated in sites where heparin is released in order to coordinate growth factor binding and activity.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant VEGF₁₆₅ was obtained from R&D Systems (Minneapolis, MN) and from the NCI Bulk Cytokine and Monoclonal Antibody Preclinical Repository (Frederick, MD). ¹²⁵I-VEGF₁₆₅ was prepared using a modified Bolton-Hunter procedure, as previously described (40). Heparin, desulfated heparin derivatives, heparin fragments, heparan sulfate, and chondroitin sulfate were from Neoparin (San Leonardo, CA), and dermatan sulfate was from Seikagaku (Japan). Heparinase I from *Flavobacterium heparinum* was from IBEX (Montreal, Canada). Plaso EpranEx™ heparin-binding plates were a gift from BMG Labtech (Sheffield, UK) and are now available from BD Biosciences. Human plasma fibronectin and α-chymotryptic fragments of human fibronectin (120 and 40 kDa) were from Chemicon International (Temecula, CA). The tryptic fragment of the N-terminal domain of human fibronectin (70 kDa) was from Sigma. Phosphate-buffered saline (PBS) without Ca²⁺ and Mg²⁺ and HEPES were purchased from Invitrogen. All other reagents used were purchased from Sigma.

Radiolabeling of Heparin—¹²⁵I-Heparin was produced using a modified Bolton-Hunter procedure. ¹²⁵I-Bolton-Hunter reagent, in anhydrous benzene (1 μCi), was dried with a gentle stream of N₂. 100 μg of heparin was added in a final volume of 30 μl in 100 mM sodium phosphate buffer, pH 8.5, and was allowed to react for 2.5 h on ice. The reaction was quenched with 200 μl of 0.2 M glycine for 45 min on ice. ¹²⁵I-Heparin was isolated using a PD-10 Sephadex G-25 M column. The specific activity of the ¹²⁵I-heparin preparation was 289 nCi/μg. ¹²⁵I-heparin was as effective as unlabeled heparin at enhancing VEGF binding to fibronectin, and isotopic dilution analysis showed that labeled and unlabeled heparin bound to fibronectin identically (data not shown).

Heparin Binding to Fibronectin and Fibronectin Fragments—Fibronectin or fibronectin fragments were adsorbed on 96-well hydrophobic polystyrene plates in PBS at 4 °C overnight (29). Incubations with ¹²⁵I-heparin were carried out in PBS at room temperature (50 μl/well). For association kinetics, incubation times were varied from 1 min to 1 h. For equilibrium binding studies, the incubation time was 2 h. Bound heparin was extracted with a 5 M NaCl, 25 mM Hepes, pH 7.5, buffer, and radioactivity was measured using a Cobra Auto-Gamma 505 counter (Packard Instruments, Meridian, CT). The concentration of bound heparin was calculated based on a 50-μl reaction volume. For dissociation kinetics studies, ¹²⁵I-heparin was incubated for 2 h at room temperature to allow for equilibrium binding, after which the ¹²⁵I-heparin solution was replaced by PBS with and without 5 μg/ml (335 nM) unlabeled heparin (release buffer). The release buffer was collected after various incubation periods at room temperature. Bound ¹²⁵I-heparin was extracted with 5 M NaCl at each time point, as described above. In competition experiments, ¹²⁵I-heparin was mixed

with 100 μg/ml unlabeled competitor: heparin, heparin fragments, chemically desulfated heparin derivatives, or other glycosaminoglycans (2-O-desulfated heparin, in which most of the O-sulfate groups on C-2 of uronic acid residues have been removed; 6-O-desulfated heparin, in which most of the O-sulfate groups on C-6 of glucosamine residues have been removed; O-desulfated heparin, in which all O-sulfate esters of heparin have been removed; N-desulfated heparin, in which N-sulfate groups of the N-sulfated glucosamine residues have been removed; heparan sulfate; N-sulfate-glucosamine-rich heparan sulfate; N-acetyl-glucosamine-rich heparan sulfate; chondroitin sulfate; and dermatan sulfate).

Kinetic Analysis of the Heparin-catalyzed Conformational Change in Fibronectin—Fibronectin, adsorbed onto hydrophobic polystyrene 96-well plates, was treated with heparin in PBS (50 μl/well) for various periods of time (1 min to 2 h) at room temperature. Following the treatment, the heparin solution was aspirated, the wells were washed three times with PBS, and VEGF binding assays were conducted with 6 nM ¹²⁵I-VEGF₁₆₅ at pH 5.5, as previously described (29). The measured concentration of bound VEGF (extracted with 5 M NaCl) and the equilibrium dissociation constant, $K_d = 100$ nM (29), were used to calculate the concentration of available VEGF binding sites on the fibronectin-coated surface, F_o ,

$$F_o = \frac{[\text{VEGF}]_b(K_d + [\text{VEGF}]_f)}{[\text{VEGF}]_f} \quad (\text{Eq. 1})$$

where $[\text{VEGF}]_b$ and $[\text{VEGF}]_f$ are the concentrations of bound and free VEGF, respectively.

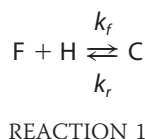
Binding of VEGF to Heparin—Heparin was adsorbed onto Plaso EpranEx™ 96-well plates. These plates have been modified by plasma polymerization to present an amine surface that can bind heparin in a noncovalent fashion (41). To determine the extent of heparin adsorption, ¹²⁵I-heparin (0.5–100 ng) was added (200 μl/well in PBS), and the amount bound was determined by extraction with a 5 M NaCl, 25 mM Hepes, pH 7.5, buffer. For the heparin concentrations tested, ~30% was adsorbed onto the plate. To determine the extent of VEGF binding to heparin, unlabeled heparin was adsorbed onto the plate, and VEGF binding assays were conducted using 6 nM ¹²⁵I-VEGF₁₆₅, as previously described (29).

Isolation and Treatment of Extracellular Matrices—ECM derived from bovine aortic endothelial cell cultures were isolated as previously described (29). Briefly, cells were maintained in 24-well plates for 3 days postconfluence and then lysed with 0.5% Triton X-100, 20 mM NH₄OH in PBS to expose the ECM surface. ECM preparations were treated with PBS (500 μl/well) with or without trypsin (0.1 μg/ml; 0.6 units/well) for 30 min at 37 °C. Following the incubation, the trypsin solution was removed, and the ECM coated wells were incubated for 10 min with a solution of soybean trypsin inhibitor in PBS (500 μl/well; 100 μg/ml) for 10 min at room temperature. The wells were washed three times with PBS (500 μl/well) prior to any further treatment (fibronectin adsorption, heparin treatment, VEGF binding, or extraction for Western blotting). The matrix preparations were visualized with Coomassie Blue after staining for 48 h at room temperature to ensure that the mild trypsinization

Catalysis of Structural Changes in Fibronectin

did not completely degrade the matrix (data not shown). Exogenous fibronectin was deposited onto the isolated matrices (40 nM in PBS; 500 μ l/well) overnight at 4 °C. Heparin treatments were conducted with 67 nM heparin (500 μ l/well) in PBS overnight at 4 °C. To visualize fibronectin content within the ECM, the matrices were extracted by scraping in 100 μ l/well of boiling sample buffer (60 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.1% bromophenol blue, 14.4 mM 2-mercaptoethanol), and samples were subjected to SDS-PAGE followed by immunoblotting using an anti-fibronectin antibody (catalog number 610077; BD Biosciences).

Kinetic Model—For the determination of the kinetic constants describing heparin binding to fibronectin, we considered the following single-site model,



where F denotes fibronectin, H is 125 I-heparin, C is the complex between fibronectin and 125 I-heparin (bound heparin), k_f is the association kinetic constant, and k_r is the dissociation kinetic constant. Dissociation of 125 I-heparin from fibronectin in the presence or absence of unlabeled heparin in solution resulted in significantly different kinetics of release (see “Results”), suggesting that rebinding events are critical for heparin-fibronectin interactions. When rebinding is included the resulting equation is as follows,

$$\frac{dp}{dt} = -k_r p + k_f H(1 - p - h) \quad (\text{Eq. 2})$$

where $p(t)$ represents the fraction of fibronectin bound to 125 I-heparin at time t , and $h(t)$ is the fraction of fibronectin bound to unlabeled heparin (when present). Assuming that unlabeled heparin equilibrates quickly, we replace h with h^* , its equilibrium value. If $p(0) \ll 1$, the resulting differential equation can be solved (42) to yield the following,

$$p(t) = p(0)e^{ct} \operatorname{erfc}(\sqrt{ct}) \quad (\text{Eq. 3})$$

where c represents a parameter describing the heparin-fibronectin interaction, which includes the diffusion coefficient, the dissociation equilibrium constant, and the surface coverage with fibronectin. Data fitting was performed using Matlab R2006b (The Mathworks, Inc., Natick, MA).

Mathematical Model Development—Our model was based on mass action kinetics and a 1:1 stoichiometry of heparin to fibronectin binding site, assuming a constant total number of binding sites. Direct independent irreversible conversion of “closed” to “open” sites was assumed. The set of nonlinear ordinary differential equations were solved using the stiff ordinary differential equation solver ode15s in Matlab R2007b with the backwards differentiation formulas option and an absolute tolerance criterion of 1×10^{-10} . The dimensionless equations describing the binding interaction are as follows,

$$\frac{dC}{d\tau} = -p_1 CH + p_2 C^* \quad (\text{Eq. 4})$$

$$\frac{dC^*}{d\tau} = p_1 CH - p_2 C^* - \frac{p_3}{p_3} C^* \quad (\text{Eq. 5})$$

$$\frac{dO^*}{d\tau} = \frac{p_2}{p_3} C^* - O^* + OH \quad (\text{Eq. 6})$$

$$\frac{dO}{d\tau} = O^* - OH \quad (\text{Eq. 7})$$

$$\frac{dH}{d\tau} = (-p_1 CH + p_2 C^* + O^* - OH)p_4 \quad (\text{Eq. 8})$$

where $C + C^* + O + O^* = 1$. C and C^* are the closed binding sites, without (C) and with (C^*) heparin bound, scaled by T , the total number of binding sites, and O and O^* are similarly scaled open binding sites. H is the heparin concentration scaled by the association constant for the open sites (K_A^O), and τ is the time scaled by the dissociation rate constant for the open sites (K_d^O). Four dimensionless parameters describe the process ($p_1 = k_a^C/k_a^O$, $p_2 = k_d^C/k_d^O$, $p_3 = k_d^C/k_c$, and $p_4 = TK_A^O/n_{av}V$), where k_a^C and K_A^O are the association rate constants for the closed and open sites, k_d^C is the dissociation rate constant for the closed sites, k_c is the conversion first order rate constant for closed to open sites, and $n_{av}V$ is a scaling value to convert concentration to binding sites based on volume. Simulations were run using $p_2 = 1$, $p_3 = 0.1$, and $p_4 = 100$ with p_1 varied to allow comparison of affinity effects. All sites were initially assumed to be in the closed conformation. The heparin concentration was varied for simulations with values shown in the figure legend.

RESULTS

Nonstoichiometric Increase in VEGF Binding to Fibronectin by Heparin—Previously (29), we showed that treatment of surface-adsorbed fibronectin with heparin leads to an increase in the number of VEGF binding sites within the fibronectin matrix, accompanied by a conformational change in fibronectin from a compact (closed) to a more extended (open) conformation (F_o). The effect of heparin was observed with fibronectin adsorbed to hydrophobic polystyrene or mica and was not dependent on whether the fibronectin was treated with heparin while being adsorbed or after surface adsorption (29).³

It is possible that heparin acts to facilitate VEGF binding either by binding to both fibronectin and VEGF to create a molecular bridge or by forming a ternary complex similar to what is observed with growth factors, such as FGF-2 and its receptors (43, 44). If so, the maximum amount of VEGF bound to fibronectin, which is equivalent to the concentration of open fibronectin binding sites (F_o) as described under “Experimental Procedures,” would be expected to be similar to the amount of heparin bound to the fibronectin matrix following the treatment period. To test these possibilities, we measured how much heparin was retained on the fibronectin matrix after the

³ M. Mitsi, K. Forsten-Williams, M. Gopalakrishnan, and M. A. Nugent, unpublished data.

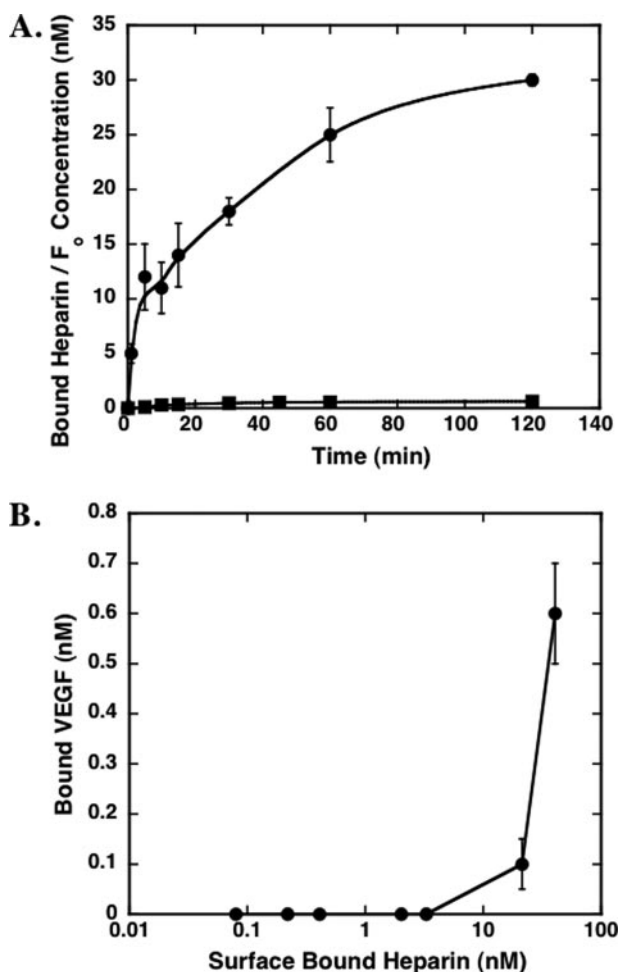


FIGURE 1. Heparin-mediated exposure of VEGF binding sites on fibronectin is not stoichiometric. A, human plasma fibronectin (40 nM), adsorbed on hydrophobic polystyrene, was treated with 67 nM heparin (^{125}I -labeled to measure bound heparin or unlabeled to conduct VEGF binding assays) for the indicated periods of time up to 2 h at room temperature. After the treatment period, the heparin solution was removed, and the concentration of heparin bound to the fibronectin matrix (\blacksquare) as well as the concentration of VEGF binding sites (\bullet) were measured. ^{125}I -VEGF binding assays were conducted with 6 nM VEGF₁₆₅, and the concentration of VEGF binding sites (F_o) was calculated as described under "Experimental Procedures." Each data point represents the mean of quadruplicate determinations \pm S.D. B, ^{125}I -heparin was bound to Plasco EpranExTM 96-well plates, and the amount of bound ^{125}I -heparin was measured by extraction with 5 M NaCl (x axis). In parallel experiments, the same amount of unlabeled heparin was adsorbed to the plate, and VEGF binding assays were conducted with 6 nM ^{125}I -VEGF at pH 5.5. Each data point represents the mean of quadruplicate determinations \pm S.D.

treatment and compared this level with the number of VEGF binding sites that are made available by the heparin treatment. Using ^{125}I -heparin, we measured the amount of heparin retained within the fibronectin matrix at various times over a 2-h treatment period. Surprisingly, we found that only a small fraction ($\sim 1\%$) of the heparin was retained on the fibronectin matrix after treatment, yet this amount of heparin resulted in a dramatic nonstoichiometric increase in the number of fibronectin molecules with open sites capable of binding VEGF (F_o) (Fig. 1A). For example, after 2 h of treatment with 67 nM heparin, only 0.62 nM remained associated with the fibronectin matrix, yet this treatment resulted in an increase in the capacity of the fibronectin to bind VEGF ($F_o = 30$ nM) such that nearly the entire population of fibronectin was available to bind VEGF.

The ratio of bound heparin to the concentration of open binding sites on fibronectin was nearly 1:50, indicating that heparin is not acting as a molecular bridge to tether VEGF to fibronectin.

Additional support for this hypothesis was provided by a direct measure of VEGF binding to heparin. This analysis revealed that the low levels of heparin that are retained on the fibronectin matrix would contribute minimally as direct binding sites for VEGF on heparin-treated fibronectin (Fig. 1B). Heparin was adsorbed onto Plasco EpranExTM heparin-binding plates, and VEGF binding to the surface-associated heparin was measured. With concentrations of adsorbed heparin up to 3 nM (5 times higher than the amount bound to fibronectin in Fig. 1A), we observed no detectable VEGF binding. Only when relatively large amounts of heparin were adsorbed onto the surface of the plates (21 and 41 nM) was detectable binding of VEGF observed. Even under these conditions, the magnitude of VEGF bound to the heparin surfaces was much less than the levels of VEGF bound to heparin-treated fibronectin. Hence, it appears that the small fraction of heparin that remains bound to fibronectin after treatment is not directly involved in mediating VEGF binding to the matrix. Indeed, removal of heparin, by passive dissociation or by active degradation with heparinase I, did not result in a reduction in the VEGF binding capacity of the heparin-treated fibronectin matrix (Fig. 2). These data indicate that once the VEGF binding sites were made available in fibronectin by exposure to heparin, the presence of heparin was no longer required to sustain the effect and that this effect is irreversible under these experimental conditions.

Heparin Binding to Fibronectin Is Rapid and Transient—The observation that heparin treatment of fibronectin can lead to an increase in VEGF binding that does not require the continued presence of heparin after the treatment period suggests that heparin catalyzes the conversion of fibronectin from a closed state to an open conformation through its ability to reversibly interact with the fibronectin matrix. The low retention of bound heparin supports a model where the interaction between heparin and fibronectin is transient. To quantify this, fibronectin was incubated with increasing concentrations of ^{125}I -heparin until equilibrium was reached, and the amount of bound heparin was measured (Fig. 3A). Determination of the number of binding sites using a single-site model showed that only a minor fraction of fibronectin was bound to heparin (~ 1 heparin molecule/20 fibronectin molecules; dissociation constant (K_d) = 19 ± 4 nM).

To further investigate the mechanism of heparin action, we performed association and dissociation kinetic analyses of heparin binding to fibronectin (Fig. 3, B and C). The association kinetics show that heparin binding approaches equilibrium by 2 h; thus, dissociation studies were conducted after allowing ^{125}I -heparin to bind to fibronectin for 2 h. Bound ^{125}I -heparin was allowed to release for various periods in buffer with and without unlabeled heparin (5 $\mu\text{g}/\text{ml}$; 335 nM). At each dissociation time point, the amount of ^{125}I -heparin remaining bound was measured (Fig. 3C). The differences observed in the kinetics of dissociation in the presence and absence of unlabeled heparin indicate that there is significant rebinding of heparin to fibronectin during the dissociation period, a function not cap-

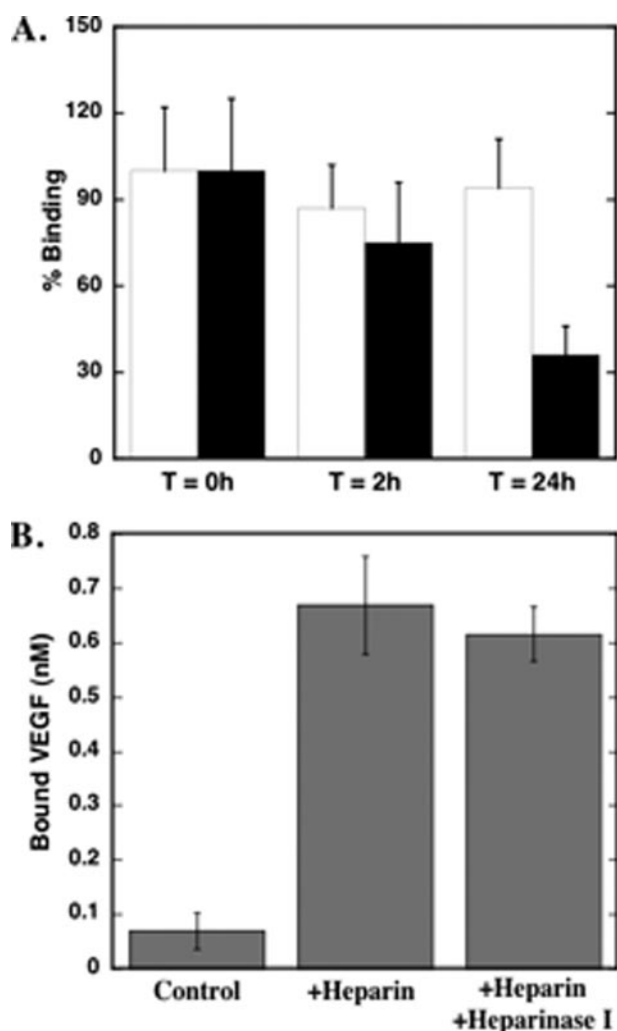


FIGURE 2. Removal of bound heparin from the fibronectin matrix does not alter VEGF binding. Human plasma fibronectin (40 nM), adsorbed on hydrophobic polystyrene, was treated with 67 nM heparin (125 I-labeled to measure bound heparin or unlabeled to conduct VEGF binding assays) for 2 h at room temperature. Following the treatment, the heparin solution was replaced with PBS (A) to allow for heparin release, and the plate was incubated at room temperature for 2 or 24 h, or it was used immediately after the removal of the heparin solution ($T = 0$). Bound heparin (black bars) and VEGF binding (white bars) were measured and normalized to their respective control values measured at the $T = 0$ point. B, after the heparin treatment, the fibronectin matrix was treated with or without heparinase I (0.01 milliunits/ml) for 6 h at 37 °C. The plates were washed to remove the heparinase I and heparin degradation products, and VEGF binding (6 nM) was measured. The data represent the mean \pm S.D. of quadruplicate determinations.

tured in the simple dissociation model. In the absence of unlabeled heparin, rebinding produced slow observed dissociation of heparin from fibronectin, and neither model (neither rebinding nor simple dissociation) fit the data. Fitting the dissociation data in the presence of unlabeled heparin to the rebinding model described under "Experimental Procedures" (Equation 3), however, was quite successful, with a single parameter fit of $c = 0.12 \pm 0.05 \text{ min}^{-1}$. A simple exponential dissociation model, where rebinding of heparin to fibronectin is not included, failed to fit the data, despite the presence of high concentrations of unlabeled heparin. The rebinding model, which fits our transient data well, would predict that a molecule of heparin that dissociates from fibronectin will rebound to the

fibronectin matrix many times prior to diffusing to the bulk solution (42).

Binding of radiolabeled heparin to fibronectin can be competed well with unlabeled heparin, whereas chemically desulfated heparin derivatives as well as other glycosaminoglycans showed reduced ability to compete with heparin for binding (Fig. 4A). The fully *O*-desulfated and 6-*O*-desulfated heparin preparations were the least effective competitors, and heparan sulfate was the most effective compared with heparin. Interestingly, heparin fragments ranging in size from 4 to 22 disaccharide units were able to compete ~ 40 –50% of the radiolabeled heparin binding (Fig. 4B). This is in contrast to the size of heparin chains required to induce the conformational change in fibronectin, as observed by an increase in VEGF binding, where fragments up to 22 sugar units long are nearly inactive (29). These results suggest that binding of heparin to fibronectin alone is not sufficient to mediate the conformational change and resulting increase in VEGF binding.

Catalytic Properties of Heparin-mediated Changes in Fibronectin—To determine whether the structural rearrangement of fibronectin initiated by heparin can proceed after heparin removal, fibronectin was treated with heparin for a short period of time (1 min), after which the heparin solution was removed, and fibronectin was incubated in buffer (PBS) for the indicated periods (Fig. 5A). The quantity of F_o was measured at each time point by conducting a VEGF binding assay. A 1-min incubation with heparin resulted in an increase in F_o (~ 2 -fold) that was not affected by the length of the post-treatment incubation, indicating that the reaction does not proceed once heparin is removed. This experiment, however, does not account for the time required to measure F_o (the 2-h VEGF binding assay), during which the reaction might continue to saturation. After a short heparin incubation, when the levels of F_o have not reached saturation, there is a possibility that the F_o levels would continue to change during the VEGF binding incubation. Therefore, the kinetics of VEGF binding to F_o would differ, depending on the duration of heparin treatment. To determine if F_o levels are changing during the VEGF binding assay period, we measured the VEGF binding kinetics after treatment of fibronectin with heparin for 0 min, 1 min, 10 min, or 1 h (Fig. 5B). Although the saturation levels of bound VEGF increased with heparin treatment time, the half-times were not different (3.4 ± 1.0 min), indicating that the amount of F_o was not changing during the VEGF binding assay incubation.

Using the VEGF binding assay to monitor the heparin-catalyzed process, we performed kinetic analysis of F_o formation with increasing concentrations of heparin: 67 nM, 670 nM, and 6.7 μM (Fig. 5C). In all cases, the reaction proceeded rapidly, reaching saturation within 1 min for the highest heparin concentration. Importantly, the final concentration of F_o generated by each heparin concentration was the same. As would be expected for a catalytic, nonreversible process, different levels of the catalyst (heparin) are able to drive the reaction to completion at different rates. In this case, the turnover of the catalyzed reaction is relatively fast, since the reaction reaches saturation within 1 h for different amounts of the catalyst (heparin) spanning a 100-fold concentration range.

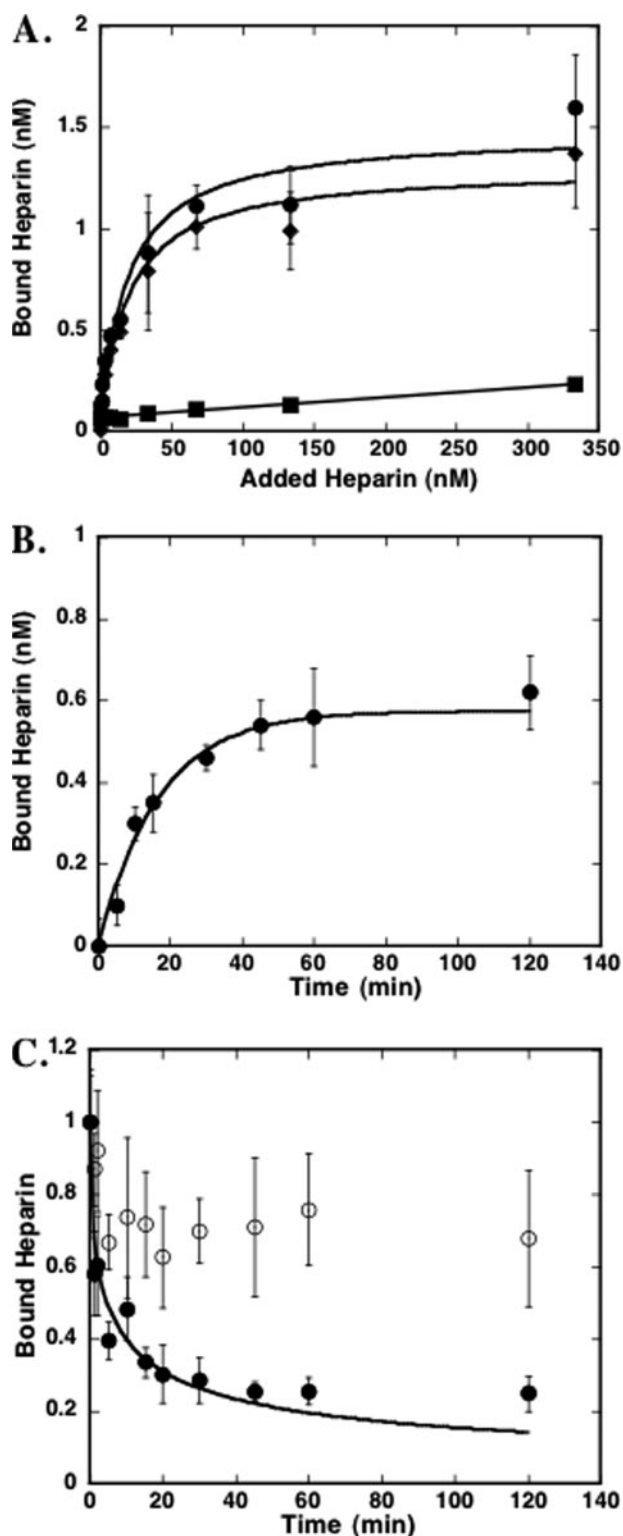


FIGURE 3. Heparin binding to fibronectin is transient. Human plasma fibronectin (40 nM) was adsorbed on hydrophobic polystyrene 96-well plates. *A*, adsorbed fibronectin was treated with increasing concentrations of ^{125}I -heparin for 2 h at room temperature. Following the treatment, bound heparin was extracted with 5 M NaCl. Nonspecific binding was determined with 100 $\mu\text{g}/\text{ml}$ unlabeled heparin; total binding (●), nonspecific binding (■), and specific binding (subtracting nonspecific from total binding) (◆) are shown. *B*, adsorbed fibronectin was treated with 67 nM ^{125}I -heparin for increasing periods of time (1 min to 2 h) at room temperature. Following the treatment, unbound heparin was removed, and bound heparin was extracted with 5 M NaCl. *C*, adsorbed fibronectin was treated with 67 nM ^{125}I -heparin for 2 h at room temperature. Following the treatment, the heparin solution was

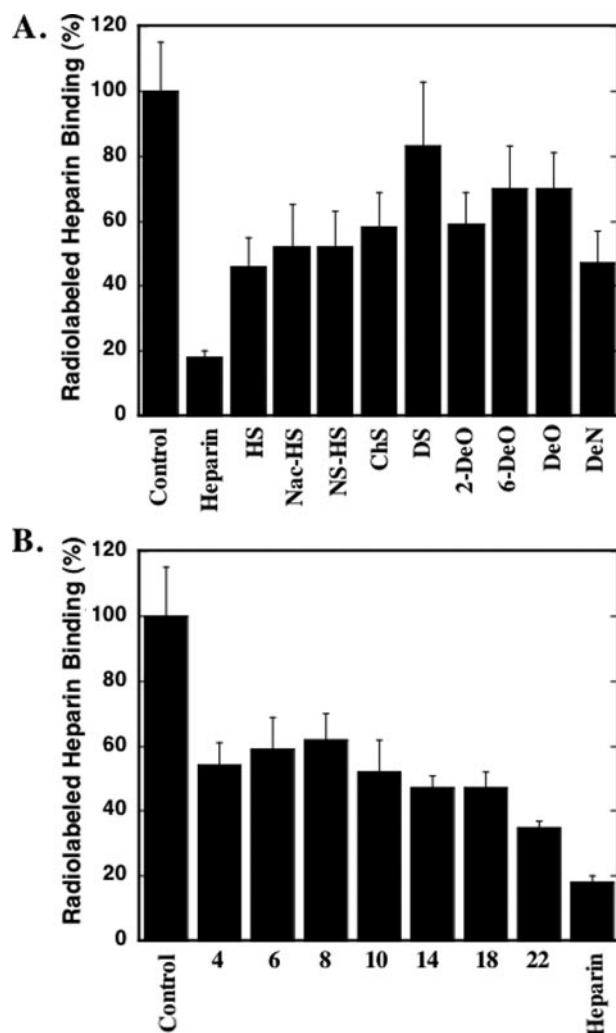


FIGURE 4. Competition of ^{125}I -heparin binding to fibronectin by heparin derivatives and fragments. Human plasma fibronectin (40 nM), adsorbed on hydrophobic polystyrene 96-well plates, was treated with ^{125}I -heparin (6.7 nM; 0.1 $\mu\text{g}/\text{ml}$) for 2 h at room temperature in the presence or absence of 100 $\mu\text{g}/\text{ml}$ unlabeled heparin or chemically desulfated heparin derivatives (*A*) (heparan sulfate (HS), *N*-acetyl-glucosamine-rich heparan sulfate (Nac-HS), *N*-sulfate-glucosamine-rich heparan sulfate (NS-HS), chondroitin sulfate (ChS), dermatan sulfate (DS), 2-*O*-desulfated heparin (2-DeO), 6-*O*-desulfated heparin (6-DeO), *O*-desulfated heparin (DeO), and *N*-desulfated heparin (DeN)) or heparin fragments (*B*) of the indicated saccharide unit length. The amount of bound heparin was measured and normalized to the level bound in the absence of competitor (100%). The data represent the mean of quadruplicate determinations \pm S.D.

The C-terminal Hep2 Domain Is the Heparin-responsive Region of Fibronectin—We have localized the heparin-responsive element that contains the VEGF binding sites to the 40-kDa C-terminal Hep2 domain of fibronectin, by conducting VEGF binding assays on three proteolytic fragments of fibronectin:

replaced with PBS with (●) or without (○) 5 $\mu\text{g}/\text{ml}$ (333 nM) unlabeled heparin, and the plate was incubated at room temperature for the indicated periods of time (1 min to 2 h). At each time point, bound heparin was extracted with 5 M NaCl. The data were normalized to the amount of bound heparin at $T = 0$. The dissociation data (*C*) were fit to the kinetic model described under "Experimental Procedures," and the equilibrium binding data (*A*) were fit to a single-site hyperbolic model: $[\text{bound heparin}] = (N[\text{free heparin}]) / ([\text{free heparin}] + K_d)$, where N represents the number of binding sites and K_d is the dissociation constant. Each data point represents the mean of quadruplicate determinations \pm S.D.

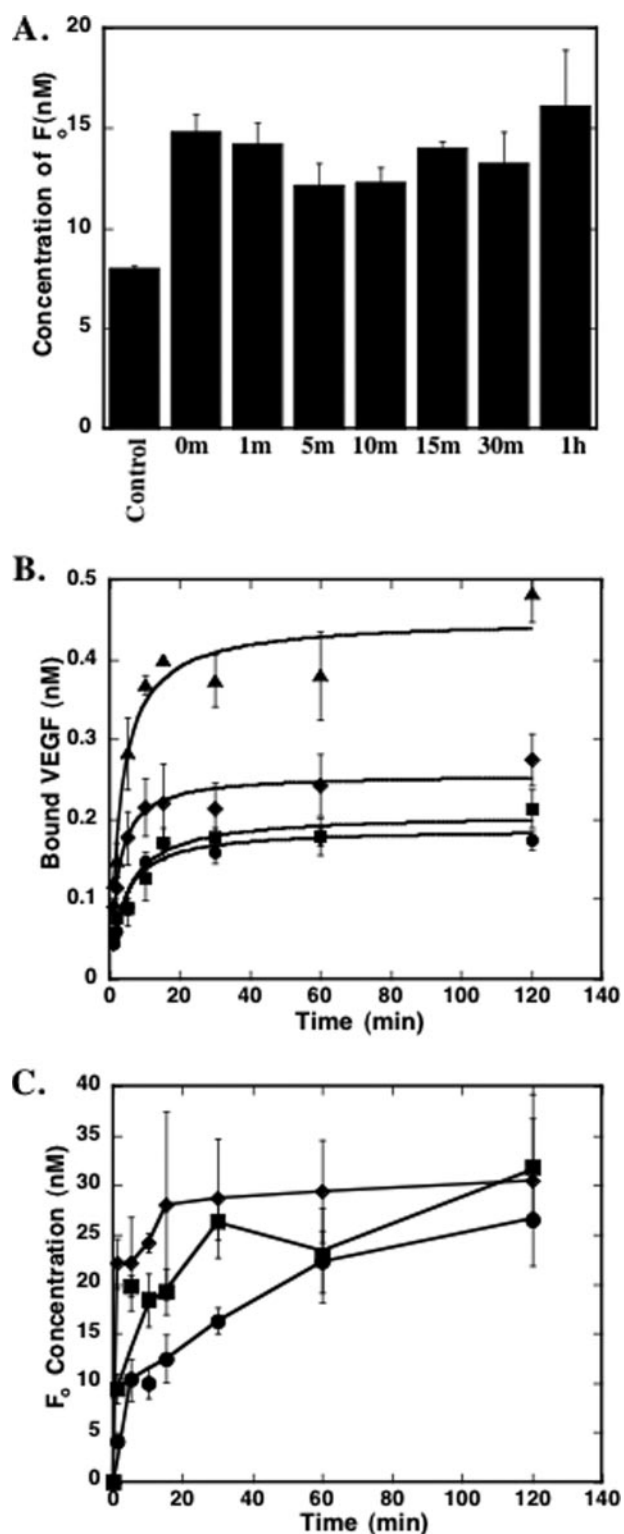


FIGURE 5. The heparin-catalyzed structural rearrangement of fibronectin does not continue upon heparin removal. Human plasma fibronectin (40 nM), adsorbed on hydrophobic polystyrene 96-well plates, was treated with 6.7 nM heparin for 1 min at room temperature, after which the heparin solution was removed and replaced with PBS, and the plate was incubated for 0 min, 1 min, 5 min, 10 min, 15 min, 30 min, or 1 h at room temperature prior to conducting a VEGF binding assay (A). The amount of bound VEGF was converted to $[F_0]$ as described under "Experimental Procedures." B, fibronectin was treated with heparin (6.7 nM) for 1 min, 10 min, or 1 h at room temperature. Following the treatment, the heparin solution was removed, and VEGF binding (6 nM 125 I-VEGF at pH 5.5) was monitored over time. ●, no heparin treatment; ■, 1-min heparin treatment; ◆, 10-min heparin treatment; ▲, 1-h

the 70-kDa N-terminal domain that contains the collagen binding site, the 120-kDa central domain that contains the RGD binding site, and the 40-kDa C-terminal domain (Fig. 6A). Heparin treatment resulted in a significant increase in VEGF binding to the 40-kDa C-terminal domain, whereas treatment of the other fragments had no effect. The effect of heparin on VEGF binding to the 40-kDa C-terminal domain could not be recapitulated with chemically desulfated heparin derivatives and heparin fragments up to 22 disaccharides long, in a fashion very similar to that observed with intact fibronectin (29) (Fig. 6B). These results suggest that the heparin-responsive VEGF binding sites are within the 40-kDa C-terminal domain of fibronectin. Consistent with these results, 125 I-heparin binding analysis with the fibronectin fragments revealed that only the 40-kDa fragment showed binding similar to that observed with intact fibronectin (Fig. 6C) (~ 1 heparin molecule/10 fibronectin 40-kDa fragment molecules; $K_d = 20 \pm 2$ nM). In addition, the association and dissociation kinetics of heparin binding to the 40-kDa C-terminal fragment were similar to those observed with intact fibronectin, with both processes being somewhat more rapid (*i.e.* dissociation parameter $c = 0.39 \pm 0.06$ min $^{-1}$; data not shown). These results suggest that cryptic VEGF binding sites are located on the 40-kDa fragment of fibronectin, and transient interactions with heparin catalyze conformational rearrangements that lead to their exposure.

Mathematical Model of Heparin Catalysis—The ability of heparin to catalyze the conversion of closed VEGF binding sites to an open conformation suggests a kinetic model whereby heparin binding to the closed Hep2 domain of fibronectin induces the rapid conversion to the open conformation, which is accompanied by a reduction in the heparin binding affinity (Fig. 7A). Based on these characteristics the process was modeled as a classic nonreversible catalytic reaction, where the catalyst (heparin) can bind to its substrate (closed fibronectin) and release its product (open fibronectin) efficiently. We constructed a mathematical model of this process to evaluate the theoretical generation of product under various conditions. We evaluated the effects of altering the relative heparin binding affinities for the open and closed conformations of fibronectin. The simulations show that when the affinity of heparin binding to the open conformation was reduced relative to that for the closed conformation, the rate of reaction (generation of open fibronectin) was dramatically increased (Fig. 7B). Simulations were also conducted where the concentration of heparin was varied, similar to what is shown experimentally in Fig. 5C, and we observed that the rate of reaction is progressively increased as the heparin concentration is increased (Fig. 7C). Together, the simulation data support the feasibility of the catalytic mechanism suggested by the experimental findings.

heparin treatment. The association kinetics data were fit to a hyperbolic model: $[VEGF] = [VEGF]_{max} / (1 + t/k_{half-time})$, where $[VEGF]_{max}$ represents the saturating levels of bound VEGF, and $k_{half-time}$ is the half-time kinetic constant. C, adsorbed fibronectin was treated with 67 nM, 670 nM, or 6.7 μ M heparin for the indicated periods of time at room temperature. Following the heparin treatment, the heparin solution was removed, and 125 I-VEGF (6 nM) equilibrium binding assays were conducted. The amount of bound VEGF was converted to concentration of open fibronectin binding sites (F_o), as described under "Experimental Procedures." Each data point represents the mean of quadruplicate determinations \pm S.D.

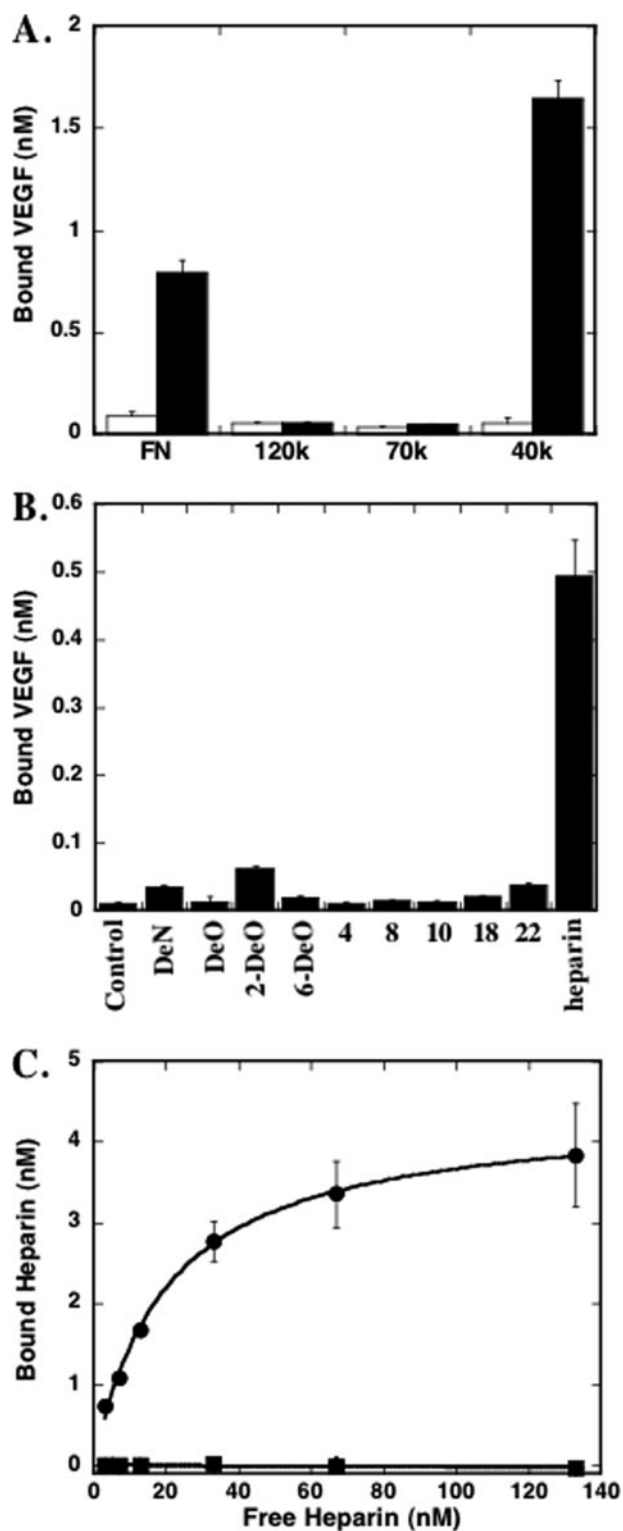


FIGURE 6. Localization of the heparin-responsive element on the 40-kDa domain of fibronectin. Human plasma fibronectin or the indicated fibronectin fragments (20 $\mu\text{g/ml}$), adsorbed on hydrophobic polystyrene 96-well plates, were incubated with 1 $\mu\text{g/ml}$ heparin (A) or the indicated chemically desulfated heparin derivatives and heparin fragments (B) for 2 h at room temperature. Following the heparin treatment, the heparin solution was removed, and ^{125}I -VEGF binding assays were conducted with 6 nM VEGF₁₆₅. In B, the adsorbed 40-kDa fragment of fibronectin was treated with heparin or the indicated modified heparin and oligosaccharides (10 $\mu\text{g/ml}$) for 2 h at room temperature prior to measuring ^{125}I -VEGF (6 nM) binding. In C, the adsorbed protein (40 nM concentration of the 40-kDa (●), 70-kDa (■), or 120-kDa (◆) fibronectin fragment) was treated with increasing concentrations of

Heparin Stimulates Binding to Fibronectin Adsorbed onto Endothelial ECM—In this study, we demonstrate that heparin can modify the binding of VEGF to fibronectin adsorbed onto polystyrene surfaces. It is possible that this process reflects the ability of heparin to alter fibronectin structure within the ECM. To test this possibility, we evaluated the ability of heparin to modify fibronectin adsorbed onto endothelial ECM (Fig. 8). Heparin treatment of endothelial ECM resulted in an ~ 2.5 -fold increase in VEGF binding. The ability of heparin to increase VEGF binding was enhanced when fibronectin was first adsorbed onto the ECM, and was decreased if the ECM was first subjected to mild trypsin digestion, which removed the endogenous fibronectin. Interestingly, the absorption of fibronectin onto trypsin-digested ECM rescued the ability of heparin to increase VEGF binding. Together, these data suggest that heparin can modify VEGF binding to fibronectin within a biological ECM.

DISCUSSION

We describe a novel catalytic process whereby heparin is able to alter the structure of fibronectin to open cryptic binding sites for VEGF. In this study, we used the heparin-mediated increase in VEGF binding to investigate the mechanism of heparin action. Treatment of fibronectin with heparin resulted in binding of a minor fraction of the added heparin, roughly equal to 1 molecule of heparin/20 molecules of fibronectin at equilibrium. Under these conditions, however, nearly all of the adsorbed fibronectin was converted to the open conformation, as indicated by its capacity to bind VEGF. Based on these observations, we ruled out a bridging mechanism where heparin interacts with both fibronectin and VEGF to bring the two proteins into close proximity, facilitating the interaction. Instead, our data indicate that heparin acts in a catalytic fashion.

The ability of heparin treatment to expose VEGF binding sites on nearly all of the available fibronectin molecules suggests that heparin is able to interact with the majority of the fibronectin molecules present. Thus, the relatively low level of heparin bound to fibronectin at steady state (~ 2 –5%) suggests that the majority of the heparin-binding sites are of too low an affinity to be observed using this method or that the treatment with heparin, which occurs as the binding reaction proceeds, alters the ability of fibronectin to bind heparin. Whereas heparin treatment results in the exposure of VEGF binding sites on fibronectin, the same conformational change might also alter the affinity or eliminate heparin-binding sites. Indeed, fibronectin in solution binds well to heparin ($>95\%$ is precipitated with heparin-agarose); however, heparin does not increase VEGF binding to globular fibronectin in solution (data not shown). Thus, the ability of heparin to induce the open conformation in fibronectin, which binds VEGF, may be coupled to a reduction in heparin affinity, and this physical rear-

^{125}I -heparin for 2 h at room temperature. Following the treatment, bound heparin was extracted with 5 M NaCl. The equilibrium binding data (C) were fit to a single-site hyperbolic model: $[\text{bound heparin}] = (N[\text{free heparin}]) / ([\text{free heparin}] + K_d)$, where N is the number of binding sites and K_d is the dissociation constant. Each data point represents the mean of quadruplicate determinations \pm S.D.

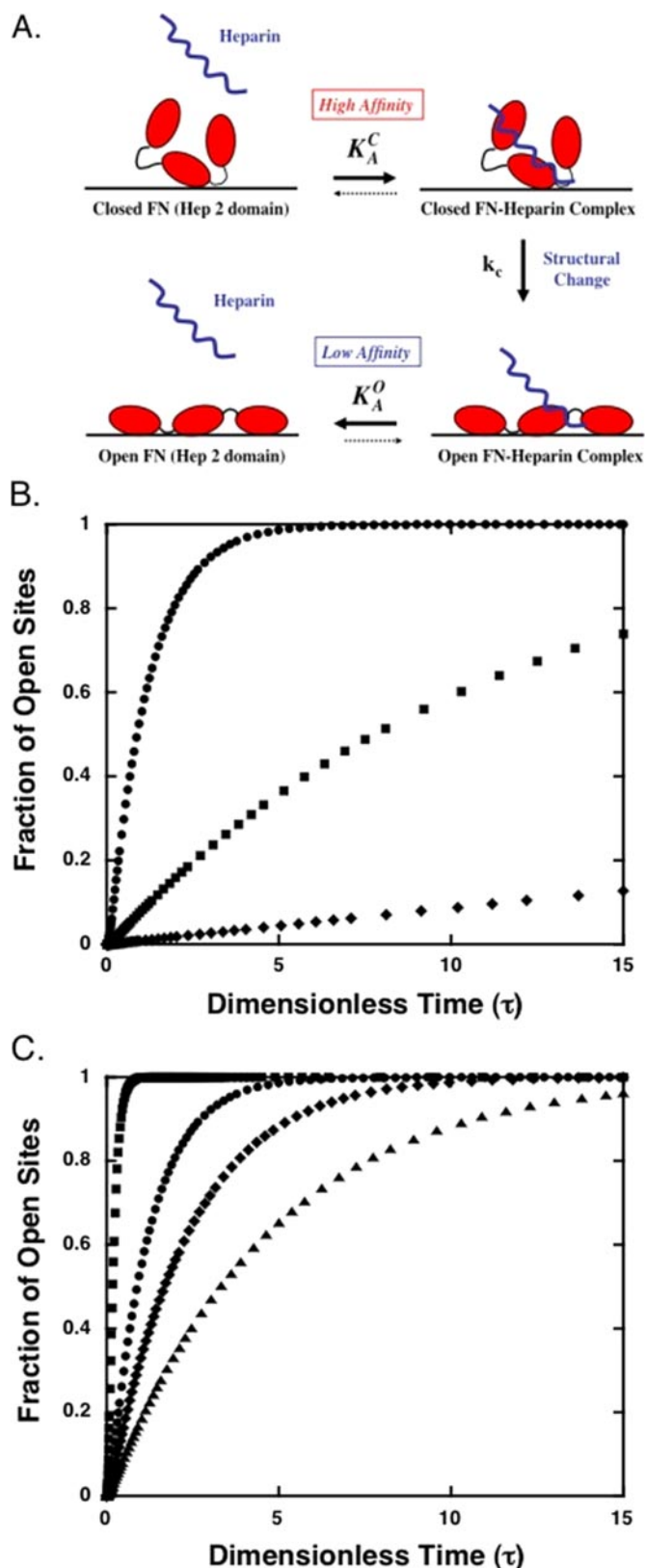


FIGURE 7. Irreversible conversion model simulates the heparin-mediated process. *A*, model schematic. The reversible binding of heparin to the Hep2 (40-kDa) domain of fibronectin in the closed conformation is defined by the affinity constant K_A^C . The rapid and nonreversible conversion of the closed Hep2 domain to the open conformation is defined by the rate constant, k_c . Reversible binding of heparin to the open conformation of the Hep2 domain is defined by the affinity constant, K_A^O . *B*, fraction of open sites (*O* and *O**) as a

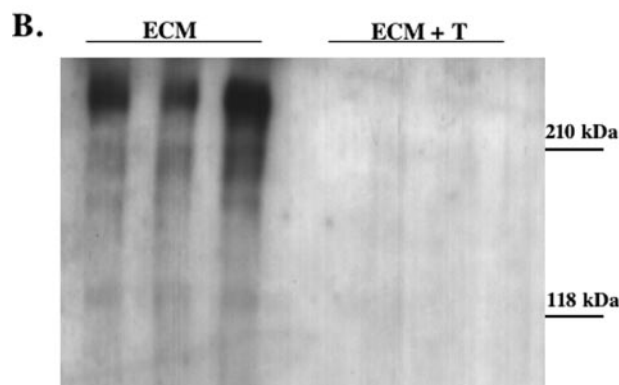
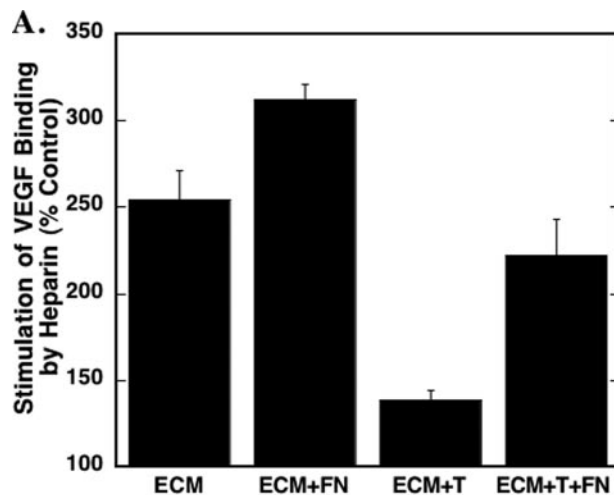


FIGURE 8. Heparin increases VEGF binding to fibronectin incorporated into endothelial cell extracellular matrix. Extracellular matrices (ECM), isolated from bovine endothelial cell cultures, were treated with or without trypsin (*ECM+T* and *ECM*, respectively). Subsequently, the matrices were incubated with and without 40 nM fibronectin (*ECM+FN* and *ECM+T+FN*), followed by an overnight treatment with or without 67 nM heparin. *A*, 125 I-VEGF binding (6 nM) to the matrices was conducted as described under "Experimental Procedures." The effect of heparin on VEGF binding is expressed as a percentage increase over control (non-heparin-treated ECM is 100%). Mean values \pm S.E. of quadruplicate measurements are shown. *B*, matrix preparations treated with or without trypsin (*ECM+T* and *ECM*) were extracted by scraping in boiling sample buffer under reducing conditions (100 μ l/well; 24-well plates). The samples were subjected to SDS-PAGE, and fibronectin was visualized by Western blotting using an anti-fibronectin antibody. Triplicate samples are shown for each condition. ANOVA followed by multicomparison *t* tests revealed significant differences between all treatment groups ($p < 0.05$).

rearrangement may require that fibronectin be in an insoluble surface-associated state.

Our theoretical simulations indicate that a reduced relative binding affinity for the open conformation would actually enhance the rate of heparin-mediated conversion of fibronectin into the open conformation (Fig. 7*B*). Hence, the reduced affinity of heparin for the open fibronectin would provide a means for the facile release of the reaction product (open fibronectin) from the catalyst (heparin), leading to catalytic turnover in a

function of dimensionless time (τ) as a function of varying the ratio of K_A^C/K_A^O , where $K_A^C = 10K_A^O$ (\bullet), $K_A^C = K_A^O$ (\blacksquare), and $0.1K_A^C = K_A^O$ (\blacklozenge). Heparin concentration (H) was $10K_A^O$. *C*, fraction of open sites as a function of heparin concentration with $K_A^C = 10K_A^O$ for $H = 1/K_A^O$ (\blacksquare), $0.1/K_A^O$ (\bullet), $0.05/K_A^O$ (\blacklozenge), and $0.025/K_A^O$ (\blacktriangle). Equations, parameters, and simulation details are as provided under "Experimental Procedures."

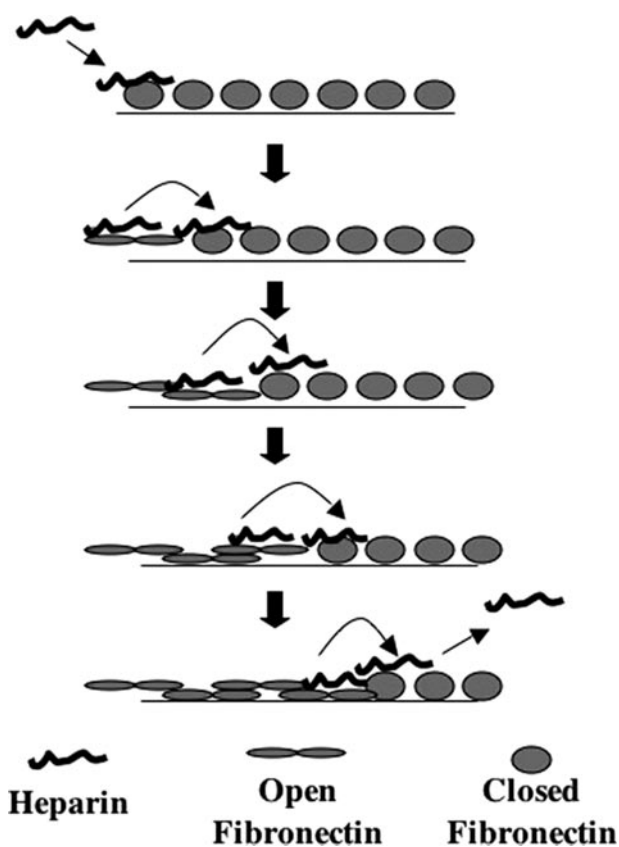


FIGURE 9. Catalytic "hopping" model for heparin-mediated changes in fibronectin conformation within the extracellular matrix. Representation of the catalytic function of heparin on fibronectin molecules immobilized in a matrix environment. Interaction between heparin and the 40-kDa domain of fibronectin (Hep2 domain) within a matrix leads to the rapid conversion of this region from a closed to open conformation, which results in an overall extension of fibronectin structure and release of heparin. Surface diffusion and frequent rebinding events enable one heparin chain to interact with and change the conformation of several fibronectin molecules before it diffuses to the bulk solution.

manner that is analogous to the mechanisms used by other biological catalysts.

Taken together, our data suggest a model where transient interactions between heparin and fibronectin are sufficient to mediate the conformational change in fibronectin that results in the generation of open VEGF binding sites (Fig. 9). Through sequential rebinding events, facilitated by reduced heparin binding affinity to open fibronectin, heparin can potentially interact with and alter the conformation of several fibronectin molecules before it eventually diffuses to the bulk solution. In this manner, heparin that has become engaged with a fibronectin-rich extracellular matrix can rapidly progress across the matrix to modify the structure of fibronectin. This type of process might be considered in terms of a reaction scheme (Fig. 7A) where the binding of heparin to fibronectin generates a heparin-fibronectin complex that leads to the irreversible conversion of fibronectin to the open conformation (catalysis step, k_c). Heparin binding to the open fibronectin product would be destabilized, leading to release and potentially transfer to a neighboring closed fibronectin molecule taking advantage of the enhanced probability of interaction through proximity. As such, the process would proceed to completion with the overall

velocity being dependent on the kinetics of heparin binding to fibronectin, the intrinsic rate of catalysis, and the kinetics of heparin hopping. Additional studies that isolate and define these discrete steps should provide the parameters required to generate quantitative kinetic models of this process to more completely evaluate its relevance in specific physiological settings (*i.e.* tissue injury, development, and inflammation).

Although the ECM is quite complex *in vivo*, our results showing that heparin treatment of endothelial ECM enhanced VEGF binding suggest that this process is active within biological systems (29) (Fig. 8). The enhanced heparin-mediated increase in VEGF binding response in the ECM samples where fibronectin was added and the loss of response in trypsin-treated ECM are consistent with this concept; however, it remains to be proven that heparin can modify endogenous fibronectin. It is also not clear to what extent this process is restricted to fibronectin.

Rebinding events in ligand-receptor interactions have been studied and reported in the literature (45, 46). Dissociation of a ligand from its receptor, diffusion in the nearby solution, and rebinding to neighboring receptors appears to be an important phenomenon in a variety of systems (47–52). Supporting the rebinding hypothesis as a means for the catalytic function of heparin, heparin-containing solutions that were recovered after treatment of fibronectin retained their capacity to alter the conformation of fibronectin even after a large number of sequential transfers to different fibronectin preparations (data not shown).

Studies on the ability of heparin to facilitate thrombin inactivation by antithrombin have also suggested that heparin possesses catalytic properties (11, 53–56). In this case, heparin appears to act as a scaffold that brings together the two proteins to facilitate the formation of the complex. In addition, binding of heparin to antithrombin III causes allosteric conformational changes that enhance its ability to inactivate thrombin as well as Factor Xa (57, 58). In the mechanism we propose here, transient interactions between heparin and fibronectin are sufficient to mediate the structural rearrangement in fibronectin. After the conformational change has taken place, the presence of heparin is no longer necessary to sustain the effect. This conclusion is supported by heparin release experiments; release of bound heparin from fibronectin does not result in a loss of VEGF binding capacity. In contrast to the thrombin-antithrombin system, the heparin-catalyzed reaction is not formally the interaction between the two target proteins (in this case VEGF and fibronectin) but rather the conformational change in fibronectin, which consequently allows for VEGF binding (Fig. 7B). At present, it is not clear how general this structural catalytic function of heparin is. It will be interesting to determine whether heparin-catalyzed conformational changes in proteins play important roles in mediating protein-protein interactions in other systems.

The ability of heparin to catalyze a conformational change in fibronectin depends on the chemical composition and size of the heparin chains. Interestingly, only very long heparin chains are able to mediate the effect (29), whereas short heparin fragments were able to compete for the binding of heparin to fibronectin. These results suggest that simple binding of heparin to fibronectin is not sufficient to catalyze the conforma-

tional change. Hence, it is possible that long heparin chains need to interact with multiple sites on fibronectin or with more than one fibronectin molecule at a time in order to mediate the effect.

We have localized the heparin-responsive element of fibronectin to the 40-kDa C-terminal fragment of fibronectin. Interestingly, under our experimental conditions, this is the only fibronectin fragment able to bind heparin. However, since our model suggests very fast interactions between heparin and fibronectin, we cannot exclude the possibility that heparin is able to transiently interact with other binding sites on fibronectin that we are unable to observe using our methods. Alternatively, binding of heparin to different domains of fibronectin may require structural elements found only within the full-length protein.

Heparin is often used as a model for interactions between proteins and heparan sulfate proteoglycans (59). There are several justifications for this approach. It is well accepted that the heparin-like segments of heparan sulfate chains are more heavily involved in interactions with target proteins due to their higher degree of sulfation and the conformational flexibility of iduronic residues (60). However, limitations in analytical techniques have made it difficult to isolate the "active" elements of heparan sulfate chain populations (61). In this context, our study could reflect important catalytic roles of heparin-like heparan sulfate regions within the extracellular matrix, especially since heparan sulfate has a similar effect on VEGF binding to fibronectin, albeit at higher concentrations (29). Alternatively, it is also possible that the mechanism we describe is unique to mast cell-derived heparin. Although the range of physiological roles of heparin remains to be fully defined, the importance of heparin secretion from mast cells in tumors has been linked to cancer metastasis and growth (7, 9, 10, 12, 14–16, 62). Hence, the ability of heparin to modulate angiogenic growth factor deposition within fibronectin could have important functional implications in cancer. Additional studies are clearly needed to evaluate these possibilities.

In the present study, we describe a process whereby heparin is able to catalytically alter the structure of the key extracellular matrix protein, fibronectin, as a possible means to coordinate VEGF bioavailability and signaling. This function might provide a connection between heparin secretion from mast cells and regulation of angiogenesis and vascular repair at sites of injury, inflammation, or malignancy.

REFERENCES

- Sasisekharan, R., and Venkataraman, G. (2000) *Curr. Opin. Chem. Biol.* **4**, 626–631
- Stringer, S. E., and Gallagher, J. T. (1997) *Int. J. Biochem. Cell Biol.* **29**, 709–714
- Metcalf, D. D., Baram, D., and Mekori, Y. A. (1997) *Physiol. Rev.* **77**, 1033–1079
- Forsberg, E., Pejler, G., Ringvall, M., Lunderius, C., Tomasini-Johansson, B., Kusche-Gullberg, M., Eriksson, I., Ledin, J., Hellman, L., and Kjellen, L. (1999) *Nature* **400**, 773–776
- Humphries, D. E., Wong, G. W., Friend, D. S., Gurish, M. F., Qiu, W. T., Huang, C., Sharpe, A. H., and Stevens, R. L. (1999) *Nature* **400**, 769–772
- Humphries, D. E., Wong, G. W., Friend, D. S., Gurish, M. F., and Stevens, R. L. (1999) *J. Histochem. Cytochem.* **47**, 1645–1646
- Ch'ng, S., Wallis, R. A., Yuan, L., Davis, P. F., and Tan, S. T. (2006) *Mod. Pathol.* **19**, 149–159
- della Rovere, F., Granata, A., Familiari, D., D'Arrigo, G., Mondello, B., and Basile, G. (2007) *Anticancer Res.* **27**, 2465–2471
- Nienartowicz, A., Sobaniec-Lotowska, M. E., Jarocka-Cyrta, E., and Lemancewicz, D. (2006) *Med. Sci. Monit.* **12**, RA53–RA56
- Azizkhan, R. G., Azizkhan, J. C., Zetter, B. R., and Folkman, J. (1980) *J. Exp. Med.* **152**, 931–944
- Nader, H. B., Lopes, C. C., Rocha, H. A., Santos, E. A., and Dietrich, C. P. (2004) *Curr. Pharm. Des.* **10**, 951–966
- Borsig, L. (2003) *Pathophysiol. Haemost. Thromb.* **33**, Suppl. 1, 64–66
- Zacharski, L. R. (2002) *Cancer Lett.* **186**, 1–9
- Zacharski, L. R., and Ornstein, D. L. (1998) *Thromb. Haemost.* **80**, 10–23
- Smorenburg, S. M., and Van Noorden, C. J. (2001) *Pharmacol. Rev.* **53**, 93–105
- Samoszuk, M., Kanakubo, E., and Chan, J. K. (2005) *BMC Cancer* **5**, 121–131
- Raman, R., Raguram, S., Venkataraman, G., Paulson, J. C., and Sasisekharan, R. (2005) *Nat. Methods* **2**, 817–824
- Powell, A. K., Yates, E. A., Fernig, D. G., and Turnbull, J. E. (2004) *Glycobiology* **14**, 17R–30R
- Potts, J. R., and Campbell, I. D. (1996) *Matrix Biol.* **15**, 313–320
- Williams, E. C., Janmey, P. A., Ferry, J. D., and Mosher, D. F. (1982) *J. Biol. Chem.* **257**, 14973–14978
- Erickson, H. P., and Carrell, N. A. (1983) *J. Biol. Chem.* **258**, 14539–14544
- Rocco, M., Carson, M., Hantgan, R., McDonagh, J., and Hermans, J. (1983) *J. Biol. Chem.* **258**, 14545–14549
- Ingham, K. C., Brew, S. A., and Atha, D. H. (1990) *Biochem. J.* **272**, 605–611
- Ingham, K. C., Brew, S. A., Migliorini, M. M., and Busby, T. F. (1993) *Biochemistry* **32**, 12548–12553
- Sachchidanand Lequin, O., Staunton, D., Mulloy, B., Forster, M. J., Yoshida, K., and Campbell, I. D. (2002) *J. Biol. Chem.* **277**, 50629–50635
- Osterlund, E., Eronen, I., Osterlund, K., and Vuento, M. (1985) *Biochemistry* **24**, 2661–2667
- Vuillard, L., Hulmes, D. J., Purdom, I. F., and Miller, A. (1994) *Int. J. Biol. Macromol.* **16**, 21–26
- Richter, H., Wendt, C., and Hormann, H. (1985) *Biol. Chem. Hoppe-Seyler* **366**, 509–514
- Mitsi, M., Hong, Z., Costello, C. E., and Nugent, M. A. (2006) *Biochemistry* **45**, 10319–10328
- Castellani, P., Viale, G., Dorcaratto, A., Nicolo, G., Kaczmarek, J., Querze, G., and Zardi, L. (1994) *Int. J. Cancer* **59**, 612–618
- Kim, S., Bell, K., Mousa, S. A., and Varner, J. A. (2000) *Am. J. Pathol.* **156**, 1345–1362
- Clark, R. A., DellaPelle, P., Manseau, E., Lanigan, J. M., Dvorak, H. F., and Colvin, R. B. (1982) *J. Invest. Dermatol.* **79**, 269–276
- Mousa, S. A., Lorelli, W., and Campochiaro, P. A. (1999) *J. Cell. Biochem.* **74**, 135–143
- Soldi, R., Mitola, S., Strasly, M., Defilippi, P., Tarone, G., and Bussolino, F. (1999) *EMBO J.* **18**, 882–892
- Tsou, R., and Isik, F. F. (2001) *Mol. Cell Biochem.* **224**, 81–89
- Miralem, T., Steinberg, R., Price, D., and Avraham, H. (2001) *Oncogene* **20**, 5511–5524
- Wijelath, E. S., Murray, J., Rahman, S., Patel, Y., Ishida, A., Strand, K., Aziz, S., Cardona, C., Hammond, W. P., Savidge, G. F., Raffii, S., and Sobel, M. (2002) *Circ. Res.* **91**, 25–31
- Wijelath, E. S., Rahman, S., Murray, J., Patel, Y., Savidge, G., and Sobel, M. (2004) *J. Vasc. Surg.* **39**, 655–660
- Goerges, A. L., and Nugent, M. A. (2004) *J. Biol. Chem.* **279**, 2307–2315
- Goerges, A. L., and Nugent, M. A. (2003) *J. Biol. Chem.* **278**, 19518–19525
- Mahoney, D. J., Whittle, J. D., Milner, C. M., Clark, S. J., Mulloy, B., Buttle, D. J., Jones, G. C., Day, A. J., and Short, R. D. (2004) *Anal. Biochem.* **330**, 123–129
- Gopalakrishnan, M., Forsten-Williams, K., Cassino, T. R., Padro, L., Ryan, T. E., and Tauber, U. C. (2005) *Eur. Biophys. J.* **34**, 943–958
- Eswarakumar, V. P., Lax, I., and Schlessinger, J. (2005) *Cytokine Growth Factor Rev.* **16**, 139–149
- Nugent, M. A., and Iozzo, R. V. (2000) *Int. J. Biochem. Cell Biol.* **32**,

- 115–120
45. Berg, H. C., and Purcell, E. M. (1977) *Biophys. J.* **20**, 193–219
 46. Lagerholm, B. C., and Thompson, N. L. (1998) *Biophys. J.* **74**, 1215–1228
 47. Goldstein, B., Posner, R. G., Torney, D. C., Erickson, J., Holowka, D., and Baird, B. (1989) *Biophys. J.* **56**, 955–966
 48. Erickson, J., Goldstein, B., Holowka, D., and Baird, B. (1987) *Biophys. J.* **52**, 657–662
 49. Erickson, J. W., Posner, R. G., Goldstein, B., Holowka, D., and Baird, B. (1991) *Biochemistry* **30**, 2357–2363
 50. Otis, T. S., Wu, Y. C., and Trussell, L. O. (1996) *J. Neurosci.* **16**, 1634–1644
 51. Lookene, A., Chevreuril, O., Ostergaard, P., and Olivecrona, G. (1996) *Biochemistry* **35**, 12155–12163
 52. Gopalakrishnan, M., Forsten-Williams, K., Nugent, M. A., and Tauber, U. C. (2005) *Biophys. J.* **89**, 3686–3700
 53. Pomerantz, M. W., and Owen, W. G. (1978) *Biochim. Biophys. Acta* **535**, 66–77
 54. Machovich, R., Bauer, P. I., Aranyi, P., Kecskes, E., Buki, K. G., and Horvath, I. (1981) *Biochem. J.* **199**, 521–526
 55. Nesheim, M. E. (1983) *J. Biol. Chem.* **258**, 14708–14717
 56. Evington, J. R., Feldman, P. A., Luscombe, M., and Holbrook, J. J. (1986) *Biochim. Biophys. Acta* **870**, 92–101
 57. Johnson, D. J., and Huntington, J. A. (2003) *Biochemistry* **42**, 8712–8719
 58. Johnson, D. J., Li, W., Adams, T. E., and Huntington, J. A. (2006) *EMBO J.* **25**, 2029–2037
 59. Coombe, D. R., and Kett, W. C. (2005) *Cell Mol. Life Sci.* **62**, 410–424
 60. Mulloy, B., and Forster, M. J. (2000) *Glycobiology* **10**, 1147–1156
 61. Sasisekharan, R., Raman, R., and Prabhakar, V. (2006) *Annu. Rev. Biomed. Eng.* **8**, 181–231
 62. Blair, R. J., Meng, H., Marchese, M. J., Ren, S., Schwartz, L. B., Tonnesen, M. G., and Gruber, B. L. (1997) *J. Clin. Invest.* **99**, 2691–2700