Thrombin Cleavage Enhances Exposure of a Heparin Binding Domain in the N-Terminus of the Fibrin \( \beta \) Chain

By Tatjana M. Odriljin, John R. Shainoff, Sarah O. Lawrence, and Patricia J. Simpson-Haidaris

Thrombin (Ila)-cleavage of fibrinogen (FBG) to form polymerized fibrin promotes endothelial cell spreading, proliferation, and von Willebrand factor release, requiring the exposure of the \( \beta 15-42 \) domain. Studies reported here indicate that ille-cleavage of fibrinopeptide B enhances exposure of a heparin binding domain at the \( \beta 15-42 \) neo-N-terminus of fibrin. Crossed immunoelectrophoresis showed heparin-induced mobility shifts indicatig complexing with FBG and with N-terminal CNBr fragments of FBG (NDSK) and of fibrin (Ila-NDSK), but no evidence of heparin complexing with FBG lacking B\( \beta 1 \)-42 or with FBG fragments D and E was seen. Elution from heparin-agarose with a linear gradient of NaCl showed that bound portions of both intact FBG and D fragments eluted below physiologic salt concentrations, whereas E\( \beta \) fragments lacking B\( \beta 1 \)-53 did not bind. NDSK bound with higher affinity than did intact FBG, whereas binding of Ila-NDSK was maximal in this system. Binding of fibrinogen to heparin agarose was saturable as well as inhabitable in a dose-dependent manner with both FBG and heparin. Scatchard analysis indicated a single class of binding site, with dissociation constants (kd) of 0.3 \( \mu \)mol/L for ille-NDSK, 0.8 \( \mu \)mol/L for NDSK, and 18 \( \mu \)mol/L for FBG. Immobilized fibrin had twofold more heparin binding sites than did immobilized FBG and required a 5.5-fold higher concentration of heparin to inhibit by 50% the binding of labeled heparin. Together, the results indicate that ille-cleavage results in enhanced exposure of two heparin binding domains (HBDs) with approximately threefold higher affinity in fibrin than in FBG. Synthetic peptide p15-42 showed highest binding to heparin-agarose followed by B\( \beta 1 \)-42, whereas peptides p18-31, p18-27, and \( \beta 24-42 \) did not bind. Thus, the primary structure of p15-42 is required for specificity of heparin binding. Basic residues within the p15-32 region segregate primarily to one side of an \( \alpha \)-helix in a helical wheel diagram, as is typical for authentic HBDs. Desulfated heparin and heparan sulfate bound more fibrinogen than did other proteoglycans; however, heparin bound sixfold more Ila-NDSK than NDSK. These results confirm that fibrin binds to heparin with higher affinity than does FBG and that fibrin binding is not solely dependent on heparin interactions of p15-42 with the negatively charged glycosaminoglycan.

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HEPARIN BINDING domains (HBDs) are found in a number of adhesive glycoproteins involved in extracellular matrix-cell interactions, including fibronectin (FN),1,2 laminin,3 thrombospondin,4,5 and von Willebrand factor.6 Specific heparin binding peptides of these glycoproteins promote cell adhesion, spreading, and focal contact assembly.7,8 Only C-terminal B\( \beta /\gamma \) chain fragments of fibrinogen (FBG) were purported to contain HBDs.8 We hypothesized that HBDs reside in the central, N-terminal domain of fibrinogen due to the clustering of basic residues within B\( \beta 1 \)-42 displaying a positive charge density similar to the HBD consensus sequence.9 Mohri et al10 were unable to detect an HBD within B\( \beta 1 \)-42, perhaps due to the presence of a V8 protease cleavage site between \( \beta \)Glu12 and \( \beta \)Ala13, the cleavage of which would destroy the integrity of a B\( \beta 1 \)-42 HBD. Therefore, we sought to determine whether the central domain of fibrinogen, particularly residues B15-42, constitutes a HBD.

The rationale for the present study was based on several observations. First, thrombin (Ila)-cleavage of FBG to form fibrin promotes endothelial cell (EC) spreading,10 proliferation,11 and von Willebrand factor release.12 These interactions require the exposure of the B15-42 domain. Second, both the primary structure13 and secondary conformation14 of B15-42 are highly conserved across species, whereas the fibrinopeptide B primary structures are not.13 Third, an EC surface glycoprotein of 130 kD was identified that binds to synthetic peptide B15-42, suggesting the presence of a receptor for fibrin on EC.14 Fourth, the C-terminal FN HBD interacts with heparan sulfate proteoglycan syndecan 4 to mediate focal adhesion formation by cells that are prespread on substrates with RGD-cell binding domains.15 Finally, \( \gamma \) thrombin cleavage of the matrix protein vitronectin (VN) alters its conformation to expose a masked HBD.16,17 Heparin has important clinical application in the prevention of thrombosis by modulating various enzymes of the clotting and fibrinolytic systems.18-25 Moreover, heparan sulfate proteoglycans are essential cofactors in receptor-growth factor interactions, cell-cell recognition systems, and cell-matrix adhesion processes.1,7,16,17,23-26 Thus, knowledge regarding the location of an HBD exposed on fibrin may have significant impact on the interpretation of studies regarding the interaction of heparin with fibrin during clotting, fibrinolysis, or fibrin-cell interactions. The data presented here support the hypothesis that fibrin B15-42 HBDs play an active role in heparin or heparan sulfate modulation of the hemostatic balance, cell-matrix interactions, and wound healing.

MATERIALS AND METHODS

Materials. Human FBG was purchased from Kabi Vitrum (Franklin, OH). Purified FBG lacking residues 1-42 of the B\( \beta \) chain (desB\( \beta 1 \)-42-FBG, also known as FBG-325) was kindly supplied by Dr A. Budzynski (Temple University, Philadelphia, PA). FBG plasmic fragments D and E were from Crystal Chemicals (Chicago,
CRYPTIC HEPARIN BINDING DOMAIN OF FIBRIN

Table 1. Structure of Different Fibrin(ogen) Fragments

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Chain Composition</th>
<th>( \gamma )-1-411</th>
<th>( \gamma )-1-78</th>
<th>( \gamma )-178</th>
<th>( \gamma )-1-82</th>
<th>( \gamma )-1-53</th>
<th>( \gamma )-406</th>
<th>( \gamma )-356</th>
<th>( \gamma )-302</th>
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</thead>
<tbody>
<tr>
<td>FBG-325</td>
<td>Ax1-610, Bx43-461</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NDSK</td>
<td>Ax1-51, Bx11-118</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ila-NDSK</td>
<td>Ax17-51, Bx15-118</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>E1</td>
<td>Ax1-104, Bx11-133</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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</tr>
<tr>
<td>E2</td>
<td>Ax20-78, Bx54-133</td>
<td>ND</td>
<td>ND</td>
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</tr>
<tr>
<td>D1</td>
<td>Ax105-208, Bx134-461</td>
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<td>ND</td>
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<td>ND</td>
<td>ND</td>
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</tr>
</tbody>
</table>

Data from Hantgan et al.29

IL). See Table 1 for a summary of the structures of fibrin(ogen) preparations and Table 2 for the fibrin(ogen)-specific monoclonal antibodies (MoAbs) used in this study. Thrombin (human plasma, 3,554 NIH U/mg) was from CalBiochem (San Diego, CA). Heparin-agarose beads (750 to 1,000 mg heparin/mL agarose, type III), heparin sodium salt (porcine intestinal mucosa, grade II, 162 USP U/mg), heparin-bovine serum albumin (BSA)-biotin, heparan sulfate sodium salt (bovine intestinal mucosa), greater than 99% de-N-sulfated heparin sodium salt, chondroitin sulfate C (shark cartilage), chondroitin sulfate A (bovine trachea), and keratan sulfate (bovine cornea) were all purchased from Sigma (St Louis, MO). Fibrinogen/bovine serum albumin (BSA)-biotin was from Bachem (Torrance, CA); peptide \( \beta \)15-42 (>99% purity) was synthesized and purified by Cornell Biotechnology Facility (Ithaca, NY); and peptides \( \beta \)11-42 (>95% purity), p18-27, and \( \beta \)M-31 (each 100% purity) were prepared as described.41

Table 2. Epitope Distribution of Fibrin(ogen) Fragments Used for CIEP and Heparin-Agarose Affinity Chromatography as Determined by ELISA and Western Blotting

<table>
<thead>
<tr>
<th>Fibrin(ogen)</th>
<th>Derivative</th>
<th>CIEP Shift Factor( ^{\circ} )</th>
<th>18C6( ^{\star} )</th>
<th>3131( ^{\star} )</th>
<th>PoAb Human FBG</th>
<th>ND</th>
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<tbody>
<tr>
<td>FBG-325</td>
<td>1.0</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>D</td>
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<tr>
<td>E2</td>
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<td>+++</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NDSK</td>
<td>1.15</td>
<td>+++</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ila-NDSK</td>
<td>1st peak</td>
<td>1.57</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>2nd peak</td>
<td>3.59</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Symbols denote the following A\textsubscript{max} ranges: --, <0.050; +, 0.100 to 0.250; ++++, >0.750.

Abbreviation: ND, not determined.

MoAbs to FBG B(1-42) (18C6) and to fibrin \( \beta \)15-42 (T2G1) were from Accurate Chemical Co (Westbury, NJ).

Anti-human FBG PoAb was obtained from Dako Corp (Carpinteria, CA).

MoAb (313) for FBG fragment D was from American Bioproducts (Pursippany, NJ).

\( ^{\circ} \)CIEP shift factor was determined from the cross-immunoelectrophoresis data shown in Fig 3A through C. The relative distance of migration (\( R_{\text{f}} \)) of the peak (center) from the origin (well) was determined by dividing the distance of peak migration by the total distance of migration of the dye front in the gel. The CIEP shift factor represents the degree of shift in anodal migration due to protein complexing with heparin, and was calculated as (\( R_{\text{f}} \) + heparin)/(\( R_{\text{f}} \) - heparin). No shift in migration in the presence of heparin results in a factor of 1.0.
Heparin-agarose affinity chromatography. Heparin-agarose columns (2.5 mL bed volume) were washed with column buffer (10 mmol/L Tris-HCl, pH 7.4). Fibrinogen (1 mL at 300 µg/mL) was applied to the column at 0.5 mL/h, washed with 12.5 mL column buffer, and eluted with a 10 mL linear gradient of 0 to 0.5 mol/L NaCl in column buffer at 20 mL/h. The conductivity of every other fraction was measured in milliosmols and then converted to millimoles per liter of NaCl. Synthetic peptides (net charge), i.e., Bβ1-42 (+1), Bβ15-42 (+3), Bβ24-42 (0), Bβ18-31 (+1), and Bβ18-27 (0), were chosen based on the charge density of basic (B) residues to other (X) residues of XBBBXXBX, as described for an HBD consensus sequence. The primary structure of Bβ1-42 is QGVNDNEEGFFS-ARGHRPLDKKREEAPSLRPAPPPISGGGYR. The HBD peptide of the B1 chain of laminin (Rα5,YVVLPRVCFEKGMNTVRiso) was chosen as a positive control HBD peptide. Peptides (5 mg/mL) were bound to heparin-agarose and eluted with a 0 to 1.0 mol/L linear gradient of NaCl.

Solid-phase binding of fibrinogen to heparin. Heparin, heparan sulfate, and desulfated heparin (20 µg/mL) were bound to polystyrene microtiter plates. After washing and blocking, FBG, NDSK, or Ila-NDSK (200 µg/mL) was added to appropriate wells (n = 3) and incubated for 2 hours at RT. Heparin bound protein was detected with 10 µg/mL PoAb anti-FBG N.

RESULTS

SDS-PAGE and ELISA. Each preparation of fibrinogen used in this study was tested to assess the degree of enzymatic (Ila, plasmin, and protease III) or chemical (CNBr) cleavage. Both FBG and FBG-325 were analyzed, reduced, by SDS-PAGE (Fig 1A) and by ELISA (Table 2). The FBG starting material was free of contaminating FN, and minimal γ-γ dimer formation was observed. Limited degradation of the Aα chain was observed, as is normally the case in pooled plasma. The Bβ chain was intact in the FBG starting material (Fig 1A and Table 2). The majority of Bβ1-42 was cleaved by protease III to produce the lower molecular weight (325 kD) FBG-325. However, about 10% of the Bβ chain was not cleaved, leaving fibrinopeptide B and Bβ1-42 sequences intact (Fig 1A and Table 2). One major band was observed for both NDSK and Ila-NDSK by SDS-PAGE (Fig 1B); however, some heterogeneity was noted. Most likely, the heterogeneity of NDSKs was due to variability in CNBr cleavage at the C-terminal boundary of the central domain, which does not alter the primary structure of the Bβ1-42 or Bβ15-42 domains. The heterogeneity of Ila-NDSK resulted additionally from incomplete Ila-cleavage in that trace amounts of fibrinopeptide B were detected with MoAb 18C6 (Table 2). Plasmin-cleaved FBG fragment D was composed of fragments D1, D2, and D3 (Fig 1B and Table 1), and ELISA of FBG E fragments with MoAbs T2G1 and 18C6 showed that the preparations were composed mostly of the late cleavage fragment E3, which is missing residues Bβ1-53 (Table 2).15

Heparin-fibrinogen complex formation. To determine whether fibrinogen formed complexes with heparin, CIEP was performed. FBG showed an anodal migratory shift in the presence of heparin, whereas FBG-325 incubated with heparin showed no migratory shift (Fig 2A and Table 2). Three precipitin lines reflecting points of equivalence were obtained with FBG-325, with a principal precipitin band of dimerically cleaved Bβ1-42 and minor bands of both cleaved/ uncleaved Bβ1-42 and dimerically uncleaved Bβ1-42. Plasmin fragments D and E1 showed no anodal migratory shift in the presence of heparin, indicating no complex formation (Fig 2B); this was most likely because fragments D and E1 lack Bβ1-42 (Table 2). Again, the multiple precipitin lines obtained with D and E are consistent with heterogeneity.
of the plasmin-cleaved FBG preparations. In the presence of heparin, Ila-NDSK showed a greater anodal migratory shift than did NDSK, suggesting that Ila-cleavage enhances the availability of a specific HBD (Fig 2C). The two peaks of precipitin line obtained in the second dimension with Ila-NDSK in the presence of heparin, coupled with the degree of shift in anodal migration, suggest a population of NDSK heterodimerically cleaved with Ila, which would leave one intact fibrinopeptide B per molecule (Table 2). Comparison of the degree of shift in anodal migration due to protein complexing with heparin indicates that fragments lacking Bβ1-42 (D, E, and FBG-325) showed no complexing with heparin (CIEP shift factor of 1.0). Fibrin(ogen) with at least one intact fibrinopeptide B showed an intermediary shift (FBG, 2.0; NDSK, 1.15); whereas homodimerically cleaved Ila-NDSK showed dramatic complexing with heparin (3.59;
Table 2). The results indicate that Bβ1-42 is required for complex formation with heparin and that IIa-cleavage enhances binding of heparin with the fibrin β15-42 N-terminus.

**Saturation and inhibition of binding of fibrinogen to heparin-agarose.** Scatchard analysis of the binding data was analyzed by the LIGAND program, which suggested a single class of binding sites (Fig 3A through C) with an average $kd$ of 0.8 μmol/L for NDSK, 18 μmol/L for FBG, and 0.3 μmol/L for IIa-NDSK. These results indicate that IIa-NDSK binds heparin with 2.7-fold higher affinity than does NDSK, but that both bind with much higher affinity than intact fibrin. The specificity of FBG binding was confirmed by the inhibition of its binding with '*'I-FBG (data not shown) and with heparin (IC50 of 42 mmol/L; Fig 3D).

**Saturation and inhibition of binding of heparin to immobilized fibrinogen.** Because intact fibrin is insoluble, $kd$ determinations as described above could not be performed. Therefore, a reciprocal binding assay was developed to measure the binding of biotin-tagged heparin to fibrin and FBG immobilized on a solid surface. Heparin-BSA-biotin bound to both immobilized FBG and fibrin in a dose-dependent and saturable manner (Fig 4A); however, maximum binding of heparin at saturation was twofold higher to fibrin than to FBG. These results indicate that IIa-cleavage of FBG resulted in exposure of twice the number of heparin binding sites. Competitive inhibition of heparin binding showed that the amount of heparin that caused a 50% inhibition in the binding of the biotin-BSA-heparin (IC50) to immobilized fibrin was 5.5-fold greater (IC50 = 6.6 μmol/L) compared with the amount required to inhibit binding to FBG (IC50 = 1.2 μmol/L; Fig 4B), suggesting higher affinity of fibrin than FBG for heparin.

**Binding of fibrinogen to heparin-agarose.** Fibrinogen was eluted from heparin-agarose with a linear gradient of 0 to 0.5 mol/L NaCl. Whereas 96.3% of Kabi FBG bound to heparin-agarose, only 40.3% of FBG-325 loaded was retained (Table 3). The differences in binding support the hypothesis that Bβ1-42 of FBG contributes to an HBD. FBG fragment D bound (56.8%) to heparin-agarose, as expected based on the localization of HBDs to both the $\beta$ and $\gamma$ chains in the D domain. In contrast, fragment E did not bind to heparin-agarose, because greater than 95% flowed through (Table 3), suggesting a requirement for the presence of Bβ1-42 to support FBG binding to heparin. Further support was obtained using the NDSKs of FBG and IIa-fibrin; greater than 95% of both NDSK and IIa-NDSK bound to heparin-agarose (Fig 5A and Table 3).

Comparison of the molarity of salt required to elute the peak fractions of each preparation showed that both FBG-325 and D fragments eluted below physiologic salt concentrations. Intact FBG eluted at below physiologic salt (120 mmol/L) as well, suggesting that FBG binding to heparin is not physiologically relevant. The reduced binding to heparin-agarose and elution of fragments D and FBG-325 with low salt indicated weak complexing of these fragments with heparin, which was borne out by the absence of detectable complex formation by CIEP (Fig 2). NDSK bound with higher affinity (200 mmol/L) than intact FBG; however, IIa-NDSK (350 mmol/L) bound to heparin-agarose with the highest affinity (Fig 5B).

**Mapping the primary structure of the fibrinogen $\beta$ chain HBD.** Laminin F9 heparin binding peptide was used to show the efficacy of the heparin-agarose column for binding peptides. Fibrin peptide β15-42 bound (96%) to heparin-agarose to a similar degree as laminin F9 peptide (96.9%).
with each peptide contributing a net +3 charge (Fig 6). FBG peptide Bβ1-42 with a lower net positive charge (+1) also bound to heparin-agarose, but to a lesser extent (67.2%). The unbound fraction of β1-42 was reapplied and none of the β1-42 bound, indicating the fraction of peptide is non-binding and that the heparin-agarose column was not overloaded. Very little β18-31 bound (8.5%) to heparin-agarose, even though this peptide has the same net charge as Bβ1-42 of +1. Furthermore, neither neutral peptide β18-27 nor β24-42 bound to heparin-agarose (<5%; Fig 6), indicating that charge is not the sole determining factor for the binding of Bβ chain N-terminal sequences to heparin. Significant binding was obtained with peptides having net charges of +3 and +1, whereas peptides with +1 net charge and neutral peptides containing basically charged Arg and Lys (β18-31 and β18-27, respectively) did not bind. We propose that residues Bβ1-42 constitute, at least in part, an FBG HBD; similarly, β15-42 constitute a fibrin HBD. Peptides β18-27, β18-31, and β24-42 did not bind to heparin-agarose (Fig 6), suggesting further that residues within β15-23, and perhaps β32-42, are essential for conferring the specificity and proper conformation on the β chain N-terminus to mediate fibrin (ogen) binding to heparin (Table 4).

Specificity of fibrin(ogen) binding to proteoglycans and glycosaminoglycans. Comparison of fibrin(ogen) binding to heparin, chondroitin sulfate, dermatan sulfate, heparan sulfate, and keratan sulfate indicated that fibrin(ogen) bound with the highest affinity to heparin (data not shown). When the binding of FBG, NDSK, and plasmin fragment D to heparin, heparan sulfate, and desulfated heparin fixed to a solid surface was compared, little difference in binding to the various heparin derivatives was observed (Fig 7). In contrast, when the binding of IIa-NDSK to all three heparin derivatives was compared, the results indicated maximal binding to heparin followed by desulfated heparin and heparan sulfate, which is sulfated to a lesser degree than heparin (Fig 7). IIa-NDSK showed fourfold to sixfold higher binding to all three heparin derivatives than was observed for FBG and for NDSK (Fig 7). Very little binding to any of
the heparin derivatives was observed with FBG fragment D in the ELISA format. Together, these results suggest that fibrin binding is specific for heparin saccharide sequences and is not solely dependent on a charge interaction of β15-42 (net charge +3) with the highly negatively charged glycosaminoglycans.

DISCUSSION

The data presented in this report clearly show that Hae-cleavage within the FBG Bβ1-42 enhances exposure of an HBD at fibrin β15-42. Previous studies have not mapped the fine point structure of the fibrinogen HBD; therefore, we performed a detailed study of the minimally relevant structure required to support binding of fibrin(ogen) to heparin. CIEP of fibrin(ogen) in the presence of heparin showed protein-glycosaminoglycan complex formation. By comparing the binding of various synthetic peptides and purified proteolytic and chemical cleavage products of fibrin(ogen) to heparin, we conclude that regions Bβ1-42 of FBG
Table 3. Comparison of the Amount of Fibrin(ogen) That Bound to the Heparin-Agarose Column to the Amount That Flowed Through the Column Based on Total Amount of Protein Recovered

<table>
<thead>
<tr>
<th>FBG Preparation</th>
<th>% Bound</th>
<th>% Not Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBG</td>
<td>96.3</td>
<td>3.7</td>
</tr>
<tr>
<td>FBG-325</td>
<td>40.3</td>
<td>59.7</td>
</tr>
<tr>
<td>NDSK</td>
<td>95.6</td>
<td>4.4</td>
</tr>
<tr>
<td>Ila-NDSK</td>
<td>96.7</td>
<td>3.3</td>
</tr>
<tr>
<td>D1.3</td>
<td>56.8</td>
<td>43.2</td>
</tr>
<tr>
<td>E3</td>
<td>4.4</td>
<td>96.6</td>
</tr>
</tbody>
</table>

and β15-42 of fibrin constitute, at least in part, the HBDs of FBG and fibrin, respectively. Mohri et al. showed that HBDs are localized to regions of the Bβ and γ chains in the C-terminal domain of FBG that are generated by V8 protease cleavage. Our data confirm the presence of these HBDs in that D fragments bound to heparin-agarose, albeit with much lower affinity, than did the NDSKs of both FBG and fibrin. The FBG Bγ HBDs differ considerably from HBDs described for other adhesive glycoproteins. The capacity of the FBG Bγ HBD to bind to heparin was not eliminated by reduction and alkylation, as would be expected. In addition, the synthetic peptide with neutral charge that corresponds to the platelet recognition domain γGQQHHLGGAKQAGDV, inhibited FBG binding to heparin-Sepharose. This peptide neither bears resemblance to the structures of other known HBD peptides nor does it share similarity to the consensus sequence described for HBDs. In contrast, the structure of β15-42 of fibrin bears considerable likeness to the HBD consensus sequence (Table 4 and Fig 8).

The strength of the complex formed between heparin and fibrin(ogen) fragments was shown to vary. Ila-NDSK bound with higher affinity than did NDSK to heparin-agarose, as shown by their elution profiles and dissociation constants. FBG E3 fragments lacking Bγ1-53 did not bind to heparin-agarose. FBG-325 lacking Bβ1-42, FBG D fragments lacking Bβ1-133, and intact FBG exhibited weaker binding to heparin-agarose because all eluted at less than 120 mmol/L NaCl. Compared with binding of FBG, tighter binding of NDSK (1.6-fold) and Ila-NDSK (2.9-fold) to heparin-agarose was observed. A comparison of the kd shows that Ila-NDSK bound to heparin-agarose with 2.7-fold higher affinity than did NDSK, with both binding with much higher affinity than intact FBG. These data are compatible with one class of binding site located on the NDSK within Bγ1-42. Also, the kd are in the same range determined by others for soluble FBG and fibrin monomer. Fibrin monomer (5.7 μmol/L) exhibited a 3.5-fold higher affinity than FBG (20 μmol/L) for heparin, consistent with our data showing that Ila-cleavage of FBG exposes a higher affinity HBD in fibrin. In addition, the low affinity of intact FBG for heparin (18 μmol/L this study; 20 μmol/L per Hogg and Jackson) suggests that the heparin binding site in the D domain is unlikely to be physiologically relevant compared with the HBD at the fibrin(ogen) Bβ chain N-terminus.

However, it is unlikely that the 30- to 60-fold enhancement of binding of fibrin(ogen) NDSKs to heparin compared

![Fig 5. Heparin-agarose affinity chromatography.](image-url)
with intact FBG reflects the in vivo situation. We hypothesize that, in vivo, the β15-42 domain of FBG is probably inaccessible to heparin/heparan sulfate proteoglycans. Subsequent IIa-cleavage and resulting conformational changes occurring during fibrin formation are most likely required to make the β15-42 site available for fibrin-cell interactions involved in hemostasis and wound repair. Unfortunately, it is extremely difficult to compare the physiologic soluble FBG to the physiologic insoluble fibrin in binding assays without experimentally manipulating the ligands so that they are either both soluble or both immobilized to a solid surface. It is known that disruption of the rigid coiled-coil region of fibrin(ogen), as would occur by CNBr cleavage, experimentally alters the native conformation by allowing more flexibility in the N-terminus. Consequently, CNBr cleavage may partially expose the β15-42 HBD in fibrin(ogen) NDSKs. Similarly, the HBD of VN is exposed by either proteolytic cleavage or chemical denaturation. Immobilization of FBG on a surface also alters native conformation by exposing neo-epitopes not accessible on FBG in solution. Further, urea treatment of FBG results in partial exposure of the conformational fibrin-β15-42 epitope recognized by T2G1, which is in close proximity to the fibrin HBD.

Fibrin monomer prepared in the presence of GPRP to inhibit polymerization was considered as an alternative ligand to Ila-NDSK for Scatchard analysis. However, the efficiency of fibrinopeptide B cleavage is accelerated as polymerization of fibrin proceeds, suggesting that polymerization-induced conformational changes facilitate IIa-release of fibrinopeptide B. Based on this interpretation, we chose not to inhibit fibrin polymerization with GPRP in case this prevented efficient release of fibrinopeptide B, a consideration crucial to our experimental objectives. In addition, when fibrinopeptide B is fully released, fibrin aggregation occurs, even in the presence of GPRP (J.R.S., unpublished data). Therefore, at the higher concentrations of protein required for the Scatchard analysis as performed in this report, the potential for fibrin monomer to become insoluble aggregates would have clouded interpretation of the data. The use of NDSK and Ila-NDSK provided soluble fragments that could never polymerize due to the absence of the complementary “a” and “b” polymerization sites found on γ chains of the D fragment.

Therefore, because it is not yet experimentally feasible to compare directly the binding affinities of the physiologic relevant forms of FBG and fibrin and most of the experiments conducted in this and other studies were performed with soluble fragments of fibrin, we developed two additional assays to measure (1) the binding of heparin to intact FBG and to fibrin immobilized to a solid surface and (2) the binding of fibrin(ogen) fragments to a panel of various

Table 4. Alignment of Fibrin β15-42 Sequence With the Heparin Binding Domain Consensus Sequence

<table>
<thead>
<tr>
<th>X</th>
<th>B</th>
<th>B</th>
<th>B</th>
<th>X</th>
<th>X</th>
<th>B</th>
<th>X*</th>
</tr>
</thead>
</table>

* The heparin binding domain consensus sequence is as defined by Cardin and Weintraub. X represents any amino acid residue and B represents basic amino acid residues.
Fig 7. Analysis of the degree of heparin sulfation on fibrin(ogen) binding. The binding of fibrin(ogen) to heparin, heparan sulfate (HS), and desulfated heparin (DSH) was measured in an ELISA format in which the heparin derivative was coated on the surface of the microtiter plate, and the fibrin(ogen) fragment bound to the heparin derivative was detected with PoAb antihuman FBG/INH. Bars represent ± 1 standard deviation.

Fig 8. Helical wheel diagram of fibrin(ogen) HBD. A helical wheel diagram was generated with Bp15-32 chain sequences according to Cardin and Weintraub. Basic residues of HBDs of antithrombin III and EC growth factor/acidic fibroblast growth factor segregate to one side of the helical face.
the ternary complex VN-IIa-antithrombin III to proteoglycans on the apical surface of EC.\textsuperscript{16} We have data indicating that fibrin fragments, but not FBG, bind to EC by a heparin-dependent mechanism involving residues β15-42 (manuscript submitted), which is compatible with the knowledge that fibrin β15-42 sequences interact directly with the EC cell surface.\textsuperscript{15} Also consistent with this is the fact that IIa-cleavage of fibrinopeptide B is required for fibrin-induced EC capillary tube formation in vitro with fibrin on the apical surface of the EC.\textsuperscript{17} Moreover, a C-terminal HBD of FN functions in focal adhesion assembly\textsuperscript{18} by interacting with a cell surface heparan sulfate proteoglycan (syndecan 4) in addition to the primary receptor:ligand interaction, which is mediated through either αβ1 or αβ3-integrins with the RGD-cell binding domain of FN.\textsuperscript{2} From these observations, we propose that fibrin(ogen) functions similarly to other heparin binding adhesive glycoproteins in cell:matrix interactions. Dual-receptor binding may promote formation of a multimolecular complex, converting an initially low-affinity interaction into a high-avidity complex. We postulate that low-affinity cell surface heparan sulfate proteoglycan may interact with the β15-42 HBD of fibrin while the integrin receptor αβ3 interacts with the fibrin(ogen) αv-chain RGD domain. Thus, the interaction of the integrin cell receptor with a matrix protein may lead to adhesion lacking focal contacts that, upon ligation of cell surface heparan sulfate proteoglycan with the HBD, could promote stable focal adhesions and stress fibers,\textsuperscript{2,19} which are indicative of cell proliferation.\textsuperscript{11} Alternatively, binding of fibrin β15-42 to the apical surface of EC (manuscript submitted), presumably via heparan sulfate proteoglycan coreceptors, may induce a migratory phenotype to promote capillary tube formation\textsuperscript{20} during angiogenesis. Together, these observations underscore the importance of elucidating the physiologic mechanism(s) that is involved in mediating fibrin-EC interactions dependent on IIa-induced exposure of the β15-42 domain.\textsuperscript{6,12,47}

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