Interaction of Fibrin(ogen) with Heparin: Further Characterization and Localization of the Heparin-Binding Site†

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ABSTRACT: The β chain 15−42 sequence of the fibrin(ogen) E region was implicated in heparin binding [Odrljin et al. (1996) *Blood 88*, 2050−2061]; whether heparin binds to other fibrin(ogen) regions remains to be clarified. To address this question, we studied the interaction of heparin with fibrinogen, fibrin, and their major fragments D_1, D-D, E_1, E_3, and αC, which together cover the entire structure of the molecule, by ligand blotting, surface plasmon resonance, and fluorescence. All three techniques revealed that at physiological ionic conditions only fibrin(ogen) and the E_1 fragment bind heparin, indicating that the only physiologically relevant heparin-binding site of fibrin(ogen) is located in its E region. To test whether the β15−42 sequence is sufficient to form this site or some additional sequences are also involved, we tested the interaction of heparin with a number of β15−42-containing fragments. The synthetic β15−42 peptide bound heparin weakly (K_d = 44.5 μM) while the recombinant β15−57 and β15−64 fragments exhibited almost 7-fold higher affinity (K_d = 6.4 and 7.1 μM, respectively), indicating that the β43−57 region is also important for heparin binding. At the same time the recombinant dimeric disulfide-linked $(\beta 15-66)_2$ fragment which mimics the dimeric arrangement of the β chains in fibrin bound heparin with high affinity (K_d = 66 nM), almost 100-fold higher than that for the monomeric fragments. This affinity was similar to those determined for fibrin and the E_1 fragment (K_d = 72 and 70 nM, respectively) suggesting that $(\beta 15-66)_2$ mimics well the heparin-binding properties of the latter two. Altogether, these results indicate that the only heparin-binding site in fibrin(ogen) is formed by NH_2-terminal portions of the β chains, including residues β15−57, and that dimerization is essential for high-affinity binding.

Fibrin(ogen) is a multifunctional plasma protein whose interaction with various proteins, cell types, and other substances including heparin and heparan sulfate provides its participation in different physiological and pathological processes. Particularly, the interaction between fibrin(ogen) and heparin was implicated in formation of a ternary complex including fibrin, heparin, and thrombin, which modulates the procoagulant activity of the latter (*1*, *2*), and in binding of fibrin(ogen) to endothelial cells through their surface proteoglycans (*3*). It was also suggested that interaction of endogenous heparin with bound fibrin(ogen) may play a role in regulation of fibrin-mediated adhesion of surfaces (*4*). Despite the physiological significance of this interaction, its underlying mechanism is not well understood.

Heparin and its structural analogue heparan sulfate are found respectively in mast cells and some hematopoietic cells (*5*, *6*) and on the surface of most cells and in the extracellular matrix (*7*, *8*). They both belong to the glycosaminoglycan family and are represented by a mixture of linear, highly sulfated, negatively charged polysaccharide chains of different length (*9*, *10*). These negative charges play an important role in interaction with clusters of positively charged residues on proteins. Heparin is probably the best studied glycosaminoglycan. Because of its ability to interact with antithrombin III and to enhance the inhibition of thrombin and some other serine proteases of the blood coagulation cascade, it is widely used as an effective anticoagulant for the prevention and treatment of coagulation disorders.

Fibrinogen consists of two identical disulfide-linked subunits, each of which is formed by three nonidentical polypeptide chains, Aα, Bβ, and γ (*11*, *12*). These chains are folded into a number of distinct domains grouped into several major structural regions, the central E region, two identical distal D regions, and the αC domains (Figure 1A). The central E region is a chemical dimer formed by the NH_2-terminal portions of all six chains; the distal D regions are formed by the COOH-terminal portions of the Bβ and γ chains and a portion of the Aα chain, and the two αC domains are made up of the COOH-terminal two-thirds of the Aα chains (*12*, *13*). The D and E regions can be separated by limited proteolysis, resulting in the E and D fragments, respectively, while the αC domains are vulnerable to proteolytic enzymes which degrade them into smaller pieces (*11*, *12*). Conversion of fibrinogen into fibrin is initiated by thrombin-mediated cleavage in the E region of two NH_2-terminal fibrinopeptides A and B (residues Aα1−16 and Bβ1−14, respectively). This is followed by spontaneous polymerization and formation of a fibrin clot.

Interaction of heparin with fibrin(ogen) was demonstrated in several studies (*1*, *4*, *14*, *15*, *16*). However, the data on localization of the heparin-binding sites are quite controver-

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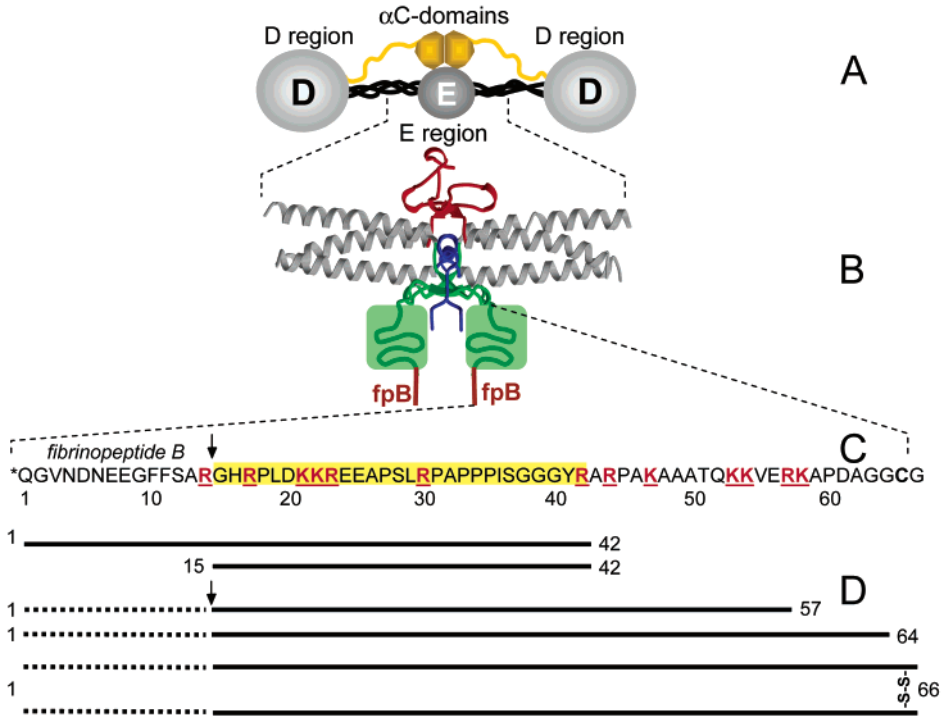

FIGURE 1: Schematic representation of fibrinogen and its central region E. Panel A represents schematically the trinodular fibrinogen molecule comprising the D-E-D regions and the αC domains. Panel B shows the ribbon diagram of the E_5 fragment based upon its crystal structure (*28*). The coiled-coil domains are colored in gray, the NH_2-terminal portions of the Aα, Bβ, and γ chains which form central domains are in blue, green, and red, respectively; the putative BβN domains (*18*) missing in the E_5 fragment are presented by the boxed green lines; fibrinopeptides B (fpB) are in brown. Panel C represents the amino acid sequence of the BβN domain starting with the pyroglutamic acid residue (*Q). The β15−42 region is highlighted by yellow; the positively charged amino acid residues are in red. The arrow indicates the thrombin cleavage site to remove fibrinopeptide B. Panel D represents schematically the β15−42-containing fragments used in this study.

sial. The first attempt to localize these sites was performed by Mohri et al. (*17*), who digested fibrinogen with V8 protease and tested the resulting fragments with heparin−Sepharose. They found two heparin-binding fragments derived from the fibrinogen D region, namely, a 40 kDa γ chain fragment and a 36 kDa β chain fragment starting with residues γ124 and β186, respectively. Odrljin et al. (*16*) also reported heparin binding in the D regions. However, in both studies, the interaction between heparin and the D fragment was detected only at low salt concentration, suggesting, as pointed out by Odrljin et al. (*16*), that it might not be physiologically relevant. At the same time these authors revealed that the NH_2-terminal disulfide knot fragment (N-DSK)[1] derived from the central E region interacted with heparin with a relatively high affinity, eluting from heparin−Sepharose above physiological ionic strength and at even higher ionic strength after removal of fibrinopeptides B with thrombin. They went on to localize the heparin-binding domains of fibrinogen and fibrin, respectively, in the 1−42 and 15−42 portions of the β chains (*16*). In contrast, Raut and Gaffney (*15*) tested the interaction of heparin with fibrinogen E and D fragments and found that only D interacts with heparin in "PBS buffer" of unspecified ionic strength. No attempts have been made to test a possible involvement of the αC domains in binding of heparin.

Thus while heparin-binding properties of the E region are established, the question of whether the D region also interacts with heparin at physiological ionic strength needs to be further addressed. In addition, although the β15−42 region was suggested to form the heparin-binding domain in fibrin (*16*), our preliminary experiments revealed that a synthetic peptide, β15−42, mimicking this region exhibited low affinity to heparin, thus raising a question of whether it represents the complete heparin-binding domain or whether other sequences are also involved in its formation. In this connection, the β15−42 region represents only part of the structure included in the putative VE-cadherin-binding βN domain (*18*) which contains some additional positively charged residues in its 43−58 region (Figure 1B,C). To address the above questions, we performed a detailed study of the interaction of heparin with fibrinogen, fibrin, and their various fragments by different techniques. We found that the E region is the only one in fibrin(ogen) which binds heparin at physiological ionic conditions, that the heparin-binding site includes residues beyond the β15−42 region, and that dimerization of the β1−57 regions is very important for the formation of the fully active heparin-binding site.

EXPERIMENTAL PROCEDURES

Proteins and Fragments. Plasminogen-depleted human fibrinogen was purchased from Enzyme Research Laboratory. Bovine α-thrombin, human plasmin, and streptavidin conjugated to alkaline phosphatase were from Sigma. The D_1 and the E_3 fragments were prepared by plasmin digestion of human fibrinogen as described earlier (*19*). The cross-

[1] Abbreviations: FA-heparin, fluoresceinamine-labeled heparin; HBS, 10 mM Hepes buffer, pH 7.4, with 150 mM NaCl; TBS, 20 mM Tris buffer, pH 7.4, with 0.15 M NaCl; SPR, surface plasmon resonance; N-DSK, NH_2-terminal disulfide knot fragment prepared by CNBr cleavage of fibrinogen.

linked dimeric D fragment (D-D) was prepared by plasmin digestion of factor XIIIa cross-linked fibrin according to the procedure described in ref *20* with some modifications (*21*). The D_1, E_3, and D-D fragments were purified from the digests by affinity chromatography on a Gly-Pro-Arg-Pro affinity column, which was prepared as described in ref *22*. The fibrinogen digest was applied to the column, and the D_1 fragment was eluted with 0.05 M Tris, pH 5.9, containing 0.25 M NaCl and 0.1 mM $CaCl_2$; the E_3 fragment was in flow-through fractions. Similarly, the fibrin digest was applied to the column, and the D-D fragment was eluted with 0.05 M Tris, pH 5.3, containing 1 M NaCl and 0.1 mM $CaCl_2$. All three fragments were further purified by size-exclusion chromatography on Sepharose 6B, equilibrated with TBS (0.02 M Tris, pH 7.4, 0.15 M NaCl) containing 0.1 mM $CaCl_2$. The E_1 fragment was obtained by dissociation of the noncovalent D-D·E_1 complex as described earlier (*21*). The human fibrinogen αC fragment (residues Aα221−610) corresponding to the αC domain was produced in *Escherichia coli* and purified as before (*23*). Soluble fibrin was prepared by mixing of human fibrinogen at 1 mg/mL in TBS containing 1 mM Ca^{2+}, with 0.1 NIH unit/mL thrombin followed by incubation at 37 °C for 30 min in the presence of 0.1 mM iodoacetamide and 5 mM Gly-Pro-Arg-Pro peptide (both from Sigma). Iodoacetamide was added to inhibit factor XIIIa, traces amounts of which are often present in fibrinogen preparations; the peptide was added to prevent polymerization of fibrin. The removal of fibrinopeptide B from fibrin was tested by Western blotting with murine mAb against fibrin neotope on the β chain (American Diagnostica Inc., Greenwich, CT).

β15−42-Containing Fragments. The β15−42-containing fragments which mimic the putative (B)βN domain and its truncated and dimeric variants (Figure 1 D) were prepared by different methods. The Bβ1−42 fragment was prepared from the plasmin digest of fibrinogen as described in ref *24*. Synthetic peptide corresponding to the β15−42 region was synthesized by SynPep (Dublin, CA). Recombinant fragments including human fibrinogen Bβ chain regions 1−57, 1−64, and 1−66 were produced in *E. coli* using pCAL-n expression vector and purified as described earlier (*18*). The disulfide-linked dimer, $(Bβ1−66)_2$, was formed from the monomeric Bβ1−66 fragment, which contains a single Cys residue at position 65, by the procedure described in ref *18*. To produce fibrinopeptide B-free species, β15−57, β15−64, and $(β15−66)_2$, the recombinant fragments were treated with thrombin and then purified as described earlier (*18*). Concentrations of the proteolytic and recombinant fragments and the synthetic peptide were determined spectrophotometrically as described in refs *18* and *21*.

Heparins. Biotinylated unfractioned heparin with an average molecular mass of 12.5 kDa, which was used in ligand blotting and in surface plasmon resonance experiments (SPR), was from Celsus Laboratories, Inc. (Cincinnati, OH). Fluoresceineamine-labeled heparin (FA-heparin) with an average molecular mass of 15 kDa was prepared by the procedure described in ref *25*.

Ligand Blotting Assay. For this study we developed a ligand blotting assay with biotinylated heparin in which the bound heparin was detected via its biotin moiety. The proteins and fragments were subjected to electrophoresis using the NuPAGE BisTris electrophoretic system (Invitrogen) and then electrotransferred to a nitrocellulose membrane (Invitrogen). To check if the transfer was successful, the membrane was stained with 0.5% Ponceau S for 1 min followed washing with water and destaining with TBS. The membrane then was blocked overnight with SuperBlock blocking buffer in TBS (Pierce) followed by incubation with 10 μg/mL biotinylated heparin in the same blocking buffer containing 0.05% Tween-20 at 4 °C for 1 h. Bound heparin was detected via its biotin moiety by reaction with streptavidin conjugated to alkaline phosphatase and visualized by using the ImmunoPure Fast Red TR/AS-MX substrate kit (Pierce) as recommended by the manufacturer. To reduce nonspecific binding of biotinylated heparin to the nitrocellulose membrane, an Avidin/Biotin blocking kit (Vector Laboratories, Inc.) was used just before incubation of a membrane with the alkaline phosphatase substrate as recommended by the manufacturer.

Surface Plasmon Resonance. The interaction of heparin with fibrinogen, fibrin, and their fragments was studied by surface plasmon resonance using the BIAcore 3000 biosensor (Biacore AB, Uppsala, Sweden) which measures association/dissociation of proteins in real time. Immobilization of biotinylated heparin to a streptavidin-coated sensor chip (Sensor Chip SA) was performed according to the procedure recommended by the manufacturer. Briefly, heparin at 100 μg/mL in HBS (0.01 M Hepes buffer, pH 7.4, 0.15 M NaCl) containing 1 mM $CaCl_2$ and 0.05% Tween-20 (binding buffer) was injected onto the chip surface at a 10 μL/min flow rate for 5 min to achieve the immobilization level of 300 response units. Binding experiments were performed in the same binding buffer and at the same flow rate. All samples were injected at 2 μM, and the association between immobilized heparin and the added proteins was monitored as the change in the SPR response; the dissociation was measured upon replacement of the ligand solution for the buffer without ligand. To regenerate the chip surface, complete dissociation of the complex was achieved by adding a solution containing 20 mM NaOH and 1 M NaCl for 1 min followed reequilibration with the binding buffer. Experimental data were analyzed using BIAevaluation 3.2 software supplied with the instrument.

Fluorescence Binding Measurements. The association of the FA-labeled heparin with fibrinogen, fibrin, and their fragments in solution was studied by monitoring the change in fluorescence anisotropy upon complex formation. The anisotropy measurements were performed with an SLM-8000C spectrofluorometer. Concentrated solutions of the proteins in TBS containing 1 mM Ca^{2+} were automatically added with a motor-driven syringe to a stirred cuvette containing FA-heparin at 0.1 μM in the same buffer while monitoring the anisotropy at 524 nm with excitation at 493 nm. The resulting concentration-dependent increase in anisotropy (A) was fitted to the equation:

$$\Delta A = \Delta A_{max}[L]/(K_d + [L]) \quad (1)$$

where K_d is the dissociation constant and [L] is the concentration of free ligand. ΔA_{max} was treated as a fitting parameter because the amount of protein added was not enough to achieve saturation. Since the concentration of FA-heparin was small compared to the K_d, the concentration of

free protein was assumed to be equal to the total concentration.

RESULTS

Since it is still unclear whether E is the only region in fibrin(ogen) that binds heparin, we tested various fragments which together cover the whole fibrin(ogen) molecule by three independent methods, ligand blotting, surface plasmon resonance (SPR), and fluorescence spectroscopy. The fragments include fibrinogen-derived D_1 and E_3, fibrin-derived D-D dimer and E_1, and the recombinant αC domain. It should be noted that both the E_1 and E_3 fragments do not contain fibrinopeptides A and B, and the major difference between them is that the β15−42 region, which was already implicated in heparin binding (*16*), is missing in the latter. We then went on to test synthetic, proteolytic, and recombinant fragments representing the amino-terminal regions of the Bβ chains in order to further localize the binding site(s) and to assess the importance of dimerization in high-affinity binding of heparin. All experiments were performed at physiological ionic strength.

Interaction between Heparin and Fibrin(ogen) Fragments Detected by Ligand Blotting. In ligand blotting experiments, fibrinogen, fibrin, and their fragments were transferred to a nitrocellulose membrane after SDS electrophoresis and probed with biotinylated heparin (Figure 2A). The experiment revealed that fibrin, which was used as a control, exhibited strong binding while fibrinogen bound heparin weakly, reinforcing the previous finding that removal of fibrinopeptides B enhances exposure of the heparin-binding site (*16*). Among all fragments tested, only E_1 exhibited strong heparin binding. Note that a relatively weak interaction with the D_1 and D-D fragments could be detected only when the nitrocellulose membrane was overexposed with the alkaline phosphatase substrates or when heparin was added at 0.1 mg/mL or higher (not shown). We conclude that, at physiological ionic strength, the E region is the only one in fibrin(ogen) which contains a strong heparin-binding site.

We next tested binding of biotinylated heparin to the individual chains of fibrinogen and fibrin by the same method (Figure 2B). The only chain which showed any binding was Bβ in fibrinogen and β in fibrin. The binding to the Bβ chain was weak and required for detection prolonged incubation of the nitrocellulose membrane with the alkaline phosphatase substrates. At the same time the β chain derived from fibrin showed a stronger response, suggesting that even when this chain is isolated from its partners, its interaction with heparin is improved by removal of fibrinopeptide B.

The fact that no binding was detected with the E_3 fragment, in which the β15−42 region is missing, reinforces the previous suggestion of Odrljin et al. (*16*) that these residues are important for binding. However, preliminary ligand blotting experiments failed to show an interaction of heparin with a synthetic β15−42 peptide, raising the question of whether additional residues beyond the β15−42 region are required for full interaction. To address this question, we prepared additional peptides containing β15−42 (Figure 1D). Among them were the fibrinogen-derived proteolytic Bβ1−42 fragment and a recombinant monomeric fragment Bβ1−57 which corresponds to the hypothetical BβN domain (*18*). We also prepared a recombinant disulfide-linked dimer

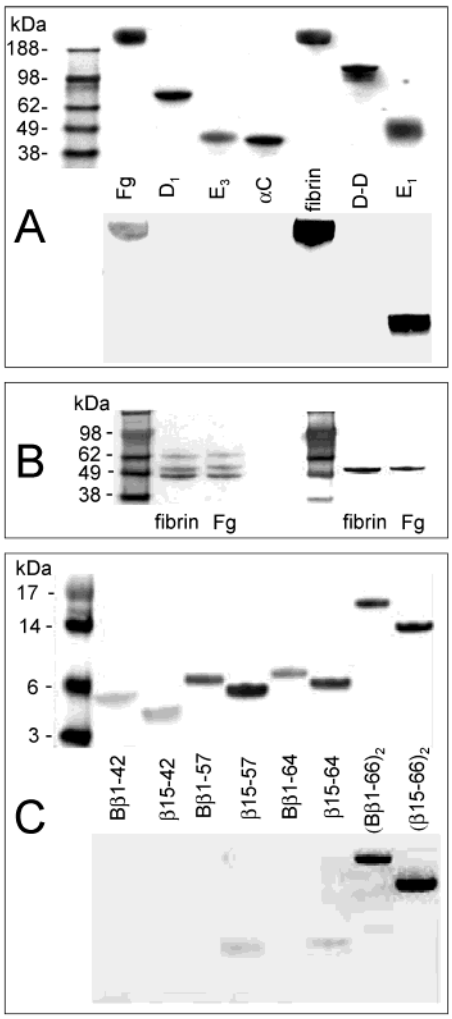

FIGURE 2: Analysis of binding of heparin to fibrinogen, fibrin, and their individual chains and fragments by ligand blot. Panel A: Fibrinogen (Fg), fibrin, and the indicated fragments were transferred to the nitrocellulose membrane after SDS electrophoresis, stained with Ponseau S (upper), and then probed with biotinylated heparin after destaining (lower). Panel B: Fibrinogen (Fg) and fibrin were electrophoresed in reduced conditions, stained with Ponseau S after being transferred to the nitrocellulose membrane (left), and then probed with biotinylated heparin after destaining (right). The binding of heparin to the Bβ chain required longer incubation with the alkaline phosphatase substrate for detection than did the β chain. Panel C: The indicated fragments were transferred to the nitrocellulose membrane after SDS electrophoresis, stained with Ponseau S (upper), and then probed with biotinylated heparin after destaining (lower). The outer left lines in all panels contain molecular mass markers (SeeBlue Plus2, Invitrogen) with indicated molecular masses.

(Bβ1−66)$_2$ to mimic the dimeric arrangement of the Bβ chains in fibrin(ogen), as well as a Bβ1−64 monomer as a control. Portions of all recombinant fragments were treated with thrombin to remove fibrinopeptides B and potentially expose their heparin-binding site(s). Ligand blotting results for these fragments are shown in Figure 2C. No binding was detected with either the proteolytic Bβ1−42 fragment or the synthetic β15−42 peptide. Longer versions of these fragments, Bβ1−57 and Bβ1−64, also failed to bind, while their thrombin-generated truncations, β15−57 and β15−64, exhibited weak binding. At the same time the dimeric fragments, both untreated and thrombin-treated, (Bβ1−66)$_2$ and (β15−66)$_2$, respectively, exhibited strong binding. These

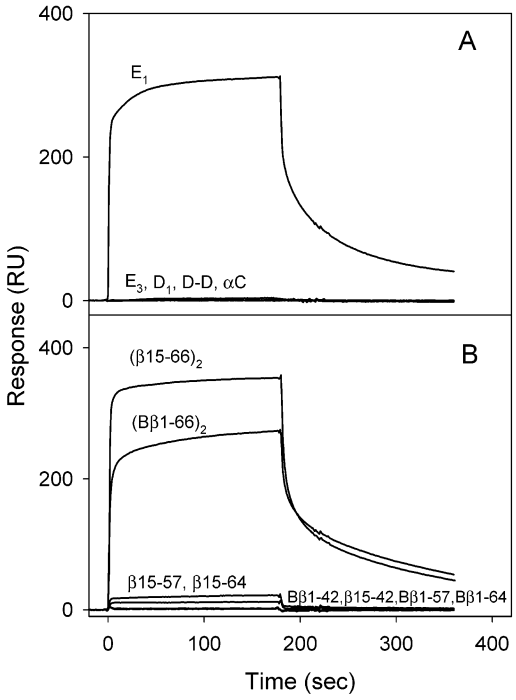

FIGURE 3: Analysis of binding of various fibrin(ogen) fragments to immobilized heparin by surface plasmon resonance. Panel A: The fibrinogen-derived D_1 and E_3 fragments, the fibrin-derived D-D and E_1 fragments, and the recombinant αC fragment, all at 2 μM, were added to the immobilized heparin, and their association/dissociation was monitored in real time while registering the resonance signal (response). Panel B: The indicated fragments, all at 2 μM, were added to immobilized heparin, and their association/dissociation was monitored in real time while registering the resonance signal (response). All experiments were performed in HBS containing 0.5 mM Ca^{2+} and 0.05% Tween-20.

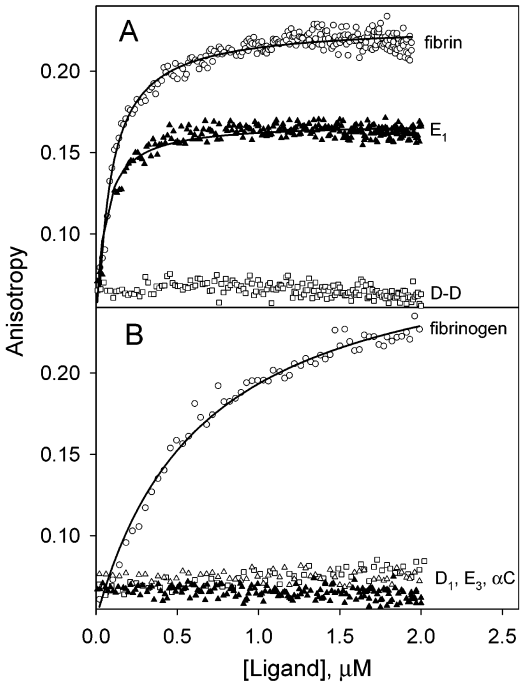

FIGURE 4: Fluorescence-detected interaction of fluoresceinamine-labeled heparin with fibrinogen, fibrin, and their fragments. FA-heparin at 0.1 μM was titrated either with Gly-Pro-Arg-Pro-solubilized fibrin and the fibrin-derived D-D and E_1 fragments (panel A) or with fibrinogen and the fibrinogen-derived D_1 and E_3 fragments and the recombinant αC fragment (panel B) while monitoring the anisotropy at 524 nm. All experiments were performed in TBS containing 0.5 mM Ca^{2+}. Solid lines represent best fits of the data to eq 1. The corresponding values of K_d are presented in Table 1.

results suggested that the β43−57 region contains additional residues that contribute to heparin binding. They also indicated that the dimeric fragments exhibited much stronger interaction with heparin than the monomeric ones. Both findings were confirmed in subsequent experiments using surface plasmon resonance.

SPR-Detected Interaction of Heparin with Fibrin(ogen) Fragments. To further test the interaction between fibrin(ogen) and its fragments with biotinylated heparin, the latter was immobilized on a streptavidin-conjugated BIAcore sensor chip. Binding of injected proteins was monitored in real time by SPR. As shown in Figure 3A, the E_1 fragment exhibited a strong interaction with immobilized heparin. The E_3, D_1, D-D, and αC fragments, all at high concentration (2 μM), exhibited no binding. Results for the β15−42-containing fragments and their dimers are shown in Figure 3B. Only the dimeric ones, $(B\beta1-66)_2$ and $(\beta15-66)_2$, exhibited strong binding to immobilized heparin; the response for β15−57 and β15−64 was very weak, and the other fragments exhibited practically no response. The higher response obtained for the truncated dimer $(\beta15-66)_2$ relative to the longer one $(B\beta1-66)_2$ at the same concentration is also consistent with the binding site being more accessible when fibrinopeptide B is removed. These results are in clear agreement with those obtained by ligand blotting. They confirm the dimeric nature of the heparin-binding site and its location near the NH_2 termini of the Bβ chains. However, attempts to quantify these interactions were complicated by variable amounts of nonspecific binding to the streptavidin-conjugated surface. We therefore turned to another method to quantify the interaction in solution.

Table 1: Dissociation Constants (K_d) for the Interaction of Fluoresceinamine-Labeled Heparin with Various Fibrin(ogen) Fragments[a]

protein or fragment	K_d	peptide	K_d
fibrinogen	228 ± 54 nM	Bβ1−42	nb
fibrin	72 ± 5 nM	β15−42	44.5 ± 3.0 μM
E_1	70 ± 10 nM	Bβ1−57	16.5 ± 1.2 μM
E_3	nb	β15−57	6.4 ± 0.1 μM
D_1	nb	Bβ1−64	15.9 ± 1.6 μM
D-D	nb	β15−64	7.1 ± 0.5 μM
αC	nb	$(B\beta1-66)_2$	210 ± 30 nM
		$(\beta15-66)_2$	66 ± 6 nM

[a] Values are means ± SD of at least three independent experiments; nb means no binding observed.

Fluorescence-Detected Interaction of Heparin with Fibrin(ogen) Fragments. To further characterize the interaction between heparin and fibrin(ogen), fluoresceinamine-labled heparin (FA-heparin) was titrated with increasing concentrations of fibrinogen, Gly-Pro-Arg-Pro-solubilized fibrin, and the various fibrin(ogen) fragments while monitoring changes in fluorescence anisotropy. As in the previous experiments, only fibrinogen, fibrin, and the E_1 fragment showed evidence of binding, manifested by a significant hyperbolic increase in anisotropy (Figure 4). The K_d values obtained by fitting the anisotropy response to a simple binding isotherm are presented in Table 1. Both the E_1 fragment and fibrin bound heparin with similar high affinity; the K_d values were found

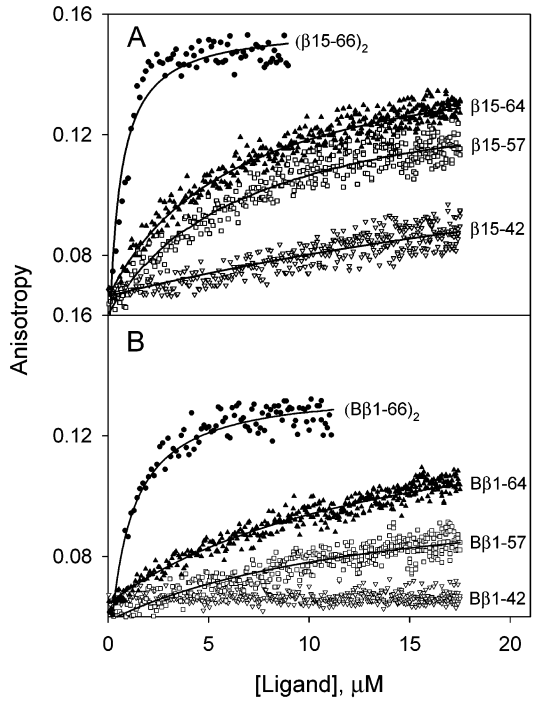

FIGURE 5: Fluorescence-detected interaction of fluoresceinamine-labeled heparin with the β15−42-containing fragments. FA-heparin at 0.1 μM was titrated with the indicated fragments mimicking fibrin (panel A) or fibrinogen (panel B) while monitoring the anisotropy at 524 nm. All experiments were performed in TBS containing 0.5 mM Ca^{2+}. Solid lines represent best fits of the data to eq 1. The corresponding values of K_d are presented in Table 1.

to be 70 and 72 nM, respectively. Such remarkable similarity of the affinities suggests that their heparin-binding sites are essentially the same. The affinity for fibrinogen (K_d = 226 nM) was more than 3-fold lower, further reinforcing the previous findings (*16*) that removal of fibrinopeptides B is important for the exposure of the heparin-binding sites. In agreement with the ligand blotting assay and SPR data, no convincing changes in anisotropy were detected when FA-heparin was titrated with the D_1, D-D, and E_3 fragments and the recombinant αC fragment. This demonstrates once again that the D regions and the αC domains of fibrin(ogen) are not involved in interaction with heparin.

All of the β15−42-containing fragments except the Bβ1−42 peptide exhibited a dose-dependent increase in anisotropy although the affinities were very different (Figure 5, Table 1). Among the monomeric fragments, β1−42 exhibited no binding and β15−42 exhibited the lowest affinity (K_d = 44.5 μM). The affinities of the longer β15−57 and β15−64 fragments were similar to each other (K_d = 6.4 and 7.1 μM, respectively) and almost 7-fold higher than that of the β15−42 fragment, confirming the importance of extra residues 43−57. These affinities were reduced more than 2-fold in the full-length Bβ1−57 and Bβ1−64 fragments, containing fibrinopeptide B. Dimerization increased the affinity for heparin by almost 2 orders of magnitude relative to the corresponding monomers while preserving the attenuating effect of fibrinopeptide B. Note that the K_d of 210 nM for $(B\beta1-66)_2$ is close to that of fibrinogen (228 nM) and the K_d of 66 nM for the shorter $(\beta15-66)_2$ is close to that of fibrin (72 nM). This suggests that the recombinant $(B\beta1-66)_2$ and $(\beta15-66)_2$ dimers mimic well the heparin-binding properties of fibrinogen and fibrin, respectively, and that their conformations may be similar to those of the corresponding fibrin(ogen) regions. Altogether, these results indicate that the heparin-binding site of fibrin(ogen) is formed by two NH_2-terminal portions of the β chains including residues from the β15−57 regions.

DISCUSSION

The present study was performed to clarify the contributions of the major fibrin(ogen) regions, D, E, and the αC domains, to heparin binding and to further localize the heparin-binding site(s). For this purpose we tested the interaction of heparin with fibrinogen, fibrin, and several proteolytic and recombinant fragments which together encompass the whole fibrin(ogen) molecule. The results obtained clearly indicate that among all studied fragments only E_1 exhibited binding to heparin at physiological ionic conditions; no interaction was observed with the D fragment or the αC fragment. These results are in good agreement with and further support the previous conclusion by Odrljin et al. (*16*) that the heparin-binding domain is located in the E region of fibrin(ogen) and that the interaction of heparin with the D region reported by others (*14*, *15*) most probably is not physiologically relevant.

The previous study by Odrljin et al. (*16*) localized the heparin-binding domain in fibrinogen and fibrin to their Bβ1−42 and β15−42 regions, respectively. Our study revealed that it includes the additional region (residues β43−57) whose presence in the monomeric β15−57 fragment increases the affinity to heparin by 7-fold in comparison with that of the β15−42 fragment. This is not surprising since this 15-residue region contains five positively charged residues, R44, K47, K53, K54, and R57 (Figure 1C), which may contribute to formation of the complete heparin-binding site, although a role for other residues cannot be excluded. In this connection, Odrljin et al. (*16*) analyzed the interaction of several peptides derived from the β15−42 region and found that although the arrangement of basic residues K21, K22, and R23 within the β15−42 region resembles heparin-binding consensus, the other residues from the 15−23 and 32−42 regions are also essential for conferring the specificity and proper folding of the heparin-binding domain. The recombinant heparin-binding fragments described in this study represent appropriate models for further mapping of the heparin-binding site in fibrinogen by site-directed mutagenesis.

Another important finding of this study is that dimerization of the β15−66 sequence dramatically enhanced its affinity to heparin; the K_d for the interaction of the dimeric $(\beta15-66)_2$ fragment with heparin was found to be more than 100-fold lower than that for the β15−64 monomer. This, together with the fact that the affinity of heparin for the dimeric fragment was comparable with that for the E_1 fragment and fibrin, indicates that the heparin-binding site is dimeric; i.e., it is formed by two NH_2-terminal portions of the β chains including residues 15−57. A similar situation has been found in our previous study of the interaction between fibrin and its endothelial cell receptor VE-cadherin, in which only the dimeric $(\beta15-66)_2$ fragment exhibited high affinity to VE-cadherin (*18*). It should be noted that the enhancement of heparin binding by protein dimerization has been described in several studies. For example, it has been demonstrated

that the affinity of monomeric interleukin-8, a proinflammatory cytokine, for heparin or heparan sulfate is too weak to allow binding at physiological ionic strength, whereas its dimeric form binds well (*26*). It has been also shown that the functional heparin-binding site of another cytokine, stromal cell-derived factor-1α, appears to be created by the dimerization of the protein which generates a positively charged crevice at the dimer interface (*27*).

By titrating fluorescently labeled heparin with fibrinogen, we obtained a value of 228 nM for the K_d. This value is in excellent agreement with that determined for the interaction between heparin and the $(B\beta1-66)_2$ dimer (K_d = 210 nM) which mimics the heparin-binding domain of fibrinogen. It also falls within the range of 137−560 nM determined by three different groups (*4*, *14*, *15*). We have no explanation for the much higher values determined by Hogg and Jackson (*1*) and by Odrljin et al. (*16*). However, it should be noted that the latter group arrived at a value of K_d = 0.8 μM for N-DSK, another fragment which also mimics the E region of fibrinogen. It should also be noted that our binding study was performed in solution at physiological ionic strength while the others used solid-phase binding assays and low or unspecified ionic strengths which could potentially influence the affinity. Altogether, these results suggest that fibrinogen interacts with heparin with a K_d that is substantially below the concentration of fibrinogen in the circulation (∼9 μM).

Our results revealed that the affinity of heparin for fibrin or the E_1 fragment is 3.2-fold higher than for fibrinogen. Moreover, the same difference in the affinity for heparin was found between the dimeric $(B\beta1-66)_2$ and $(\beta15-66)_2$ fragments which mimic fibrinogen and fibrin, respectively. This means that the fibrinopeptides interfere with binding of heparin. Fibrinopeptide B has three negative charges which could introduce some repulsion for the anionic polymer. Alternatively, the NH_2-terminal region of the Bβ chain in fibrinogen could interact with other regions to partially mask the binding site. This would imply some tertiary structure within the βN domains, structure that has yet to be demonstrated (*18*). In any case, our findings agree with those of Odrljin et al. (*16*), whose N-DSK fragment bound heparin with 2.7-fold higher affinity after treatment with thrombin to remove the fibrinopeptides. Hogg and Jackson (*1*) also observed a 3.5-fold difference in affinity of heparin for fibrinogen and fibrin, although, as mentioned above, their K_ds were much higher than those determined here and in refs *4*, *14*, and *15*. In any case, it seems clear that conversion of fibrinogen into fibrin results in about a 3-fold increase of its affinity for heparin.

In summary, the results of this study clearly indicate that the major, and probably the only, physiologically relevant heparin-binding site in fibrin(ogen) is located in its central region E, that this site is formed by the amino-terminal regions of two Bβ chains including residues 15−57, and that a dimeric arrangement of these regions is critical for the formation of the fully active heparin-binding site. The dimeric $(B\beta1-66)_2$ and $(\beta15-66)_2$ fragments, which mimic well the heparin-binding properties of fibrinogen and fibrin, respectively, should prove useful in further site-directed mutation studies of the interaction with heparin.

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