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Evidence That Binding to the Carboxyl-Terminal Heparin-Binding Domain (Hep II) Dominates the Interaction between Plasma Fibronectin and Heparin†

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ABSTRACT: We assessed the participation of the three known heparin-binding domains of PFn (Hep I, Hep II, Hep III) in their interaction with heparin by making a quantitative comparison of the fluid-phase heparin affinities of PFn and PFn fragments under physiologic pH and ionic strength conditions. Using a fluorescence polarization binding assay that employed a PFn affinity-purified fluorescein-labeled heparin preparation, we found that >98% of the total PFn heparin-binding sites exhibit a K_d in the 118–217 nM range. We also identified a minor (<2%) class of binding sites exhibiting very high affinity ($K_d \sim 1$ nM) in PFn and the carboxyl-terminal 190/170 and 150/136 kDa PFn fragments. This latter activity probably reflects multivalent inter- or intramolecular heparin-binding activity. Amino-terminal PFn fragments containing Hep I (72 and 29 kDa) exhibited low affinity for heparin under physiologic buffer conditions ($K_d \sim 30\,000$ nM). PFn fragments (190/170 and 150/136 kDa) containing both the carboxyl-terminal Hep II and central Hep III domains retained most of the heparin-binding activity of native PFn (K_d = 278–492 nM). The isolated Hep II domain (33-kDa fragment) exhibited appreciable, but somewhat lower (2–5-fold), heparin affinity compared to the 190/170-kDa PFn fragment. Heparin binding to the 100-kDa PFn fragment containing Hep III was barely detectable ($K_d > 30\,000$ nM). From these observations, we conclude that PFn contains only one major functional heparin-binding site per subunit, Hep II, that dominates the interaction between heparin and PFn.

Plasma fibronectin (PFn)[1] is a large soluble heterodimeric glycoprotein found in blood (Mosesson & Umfleet, 1970; Mosesson et al., 1975; Yamada, 1983). An insoluble "matrix" form of fibronectin (Fn) is also deposited in extracellular matrices and basement membranes. These proteins have been shown to mediate a variety of adhesive biological functions such as cell attachment and spreading. The various adhesive properties are correlated with the interaction of the various Fn binding domains with macromolecules found within cells, on cell surfaces, and in extracellular matrices.

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[1] Abbreviations: PFn, plasma fibronectin; Fn, fibronectin; PMSF, phenylmethanesulfonyl fluoride; PEG, poly(ethylene glycol); TBS, Tris-buffered saline; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; Fl-heparin, fluorescein-labeled heparin; M_r, average molecular weight; K_d, dissociation constant; kDa, kilodaltons; FP, fluorescence polarization; ND, not determined; μ, ionic strength.

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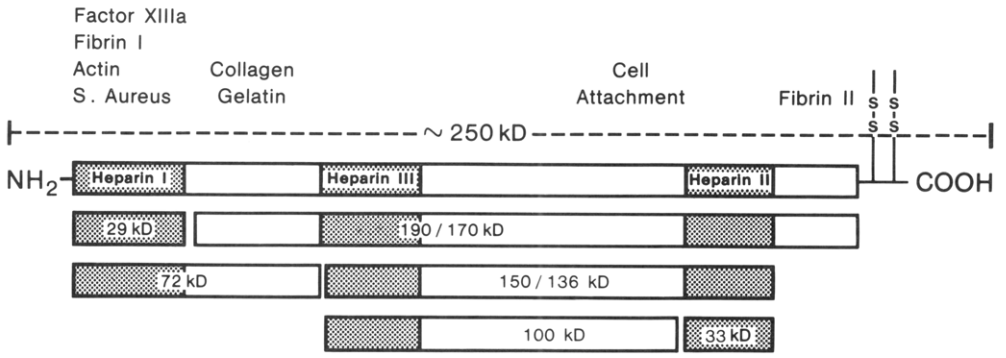

FIGURE 1: Diagram illustrating the respective locations of the PFn heparin-binding domains (Smith & Furcht, 1982; Gold et al., 1983) and the structural origin of the various proteolytic PFn fragments employed in this study. The location of the other various ligand-binding domains on the PFn subunit is based on literature reviewed previously (Yamada, 1983). The complete 2325 amino acid sequence of the PFn subunit has been reported (Kornblihtt et al., 1985). In this sequence, positions 1, 260, 873, 1218, 1600, and 2034 mark the beginnings of the Hep I, collagen/gelatin, Hep III, cell attachment, Hep II, and fibrin II domains, respectively (Kornblihtt et al., 1985). The 190/170- and 100-kDa PFn fragments begin at positions 260 and 589, respectively (Homandberg & Erickson, 1986). The sequence of the first 13 amino acids of the 150/136-kDa PFn fragment corresponded to positions 688–701 in the PFn sequence. Preparations of the 33-kDa PFn fragment yielded two overlapping sequences corresponding to positions 1578–1595 and 1583–1602, respectively. The amino-terminal ends of both the 29- and 72-kDa PFn fragments are blocked, as is native PFn (Mosesson et al., 1975; Homandberg & Erickson, 1986). Heterogeneity of the 190/170- and 150/136-kDa fragments presumably arises from the size difference between the two types of "A" and "B" PFn subunits (Click & Balian, 1985).

The specific mechanism by which sulfated glycosaminoglycans (e.g., heparin, heparan sulfate) interact with the various heparin-binding sites on PFn is not understood. At least three distinguishable heparin-binding domains (Figure 1) have been localized on each PFn subunit (Hayashi & Yamada, 1982; Smith & Furcht, 1982; Gold et al., 1983). The Hep I and Hep II domains are located at the amino- and carboxyl-terminal ends of the molecule, within segments of 27 and 31 kDa, respectively (Smith & Furcht, 1982). The Hep III domain has been localized to a more central region of each subunit within a segment of 30 kDa (Gold et al., 1983). PFn fragments containing the isolated Hep I, Hep II, and Hep III domains exhibit intermediate, high and low affinity, respectively, for heparin–Sepharose affinity resins (Gold et al., 1983). Previous studies employing solid-phase binding assays revealed that both chick embryo fibroblast Fn (Yamada et al., 1980) and PFn (Tarsio et al., 1987) contained at least two unique classes of heparin-binding sites exhibiting dissociation constants in the 4–200 nM range. Similar results were reported (K_ds = 35, 1000 nM) when fluid-phase PFn affinity for heparin was evaluated by fluorescence polarization (Bentley et al., 1985a).

Heparin (Welsh et al., 1983) and heparan sulfate (Österlund et al., 1985) reportedly induce changes in PFn secondary structure. PFn exhibits enhanced binding activity for denatured collagen (gelatin) in the presence of heparin (Johansson & Höök, 1980; Ruoslahti & Engvall, 1980) and dextran sulfate (Ruoslahti et al., 1979). Heparin also induces precipitation of PFn in the cold (Stathakis & Mosesson, 1977). This cold-insoluble precipitate is believed to be a multimeric PFn aggregate (Richter et al., 1985) that displays enhanced affinity for native collagen (Jilek & Hörmann, 1979) and macrophages (Hörmann & Jelinic, 1980). In order to further understand the structural features responsible for PFn heparin-binding activity, we assessed the respective participation of its three heparin-binding domains. We accomplished this by using a fluorescence polarization binding assay (Bentley et al., 1985b) to make a quantitative comparison of the fluid-phase heparin affinities of PFn and proteolytic PFn fragments under physiologic pH and ionic strength conditions.

Materials and Methods

Outdated human plasma was obtained from the Blood Center of Southeastern Wisconsin (Milwaukee, WI). Heparin (164 USP units/mg), gelatin, cathepsin D, and PMSF were obtained from Sigma (St. Louis, MO). Acetic anhydride, PEG ($\bar{M}_r$ 15 kDa), and cyanogen bromide were purchased from Aldrich (Milwaukee, WI). Electrophoresis supplies and the Bradford protein dye reagent were obtained from Bio-Rad (Richmond, CA). Trasylol was obtained from FBA Pharmaceuticals (New York, NY). α-Thrombin was a generous gift from Dr. John Fenton (New York State Department of Health, Albany, NY). Heparin was N-acetylated by acetic anhydride treatment (Means & Feeney, 1971). All other common chemicals were obtained from Fisher (Pittsburgh, PA).

Sepharose 4B-CL and Ultrogel AcA44 were obtained from Pharmacia (Piscataway, NJ) and LKB (Rockville, MD), respectively. Heparin– and gelatin–Sepharose 4B-CL affinity resins were prepared by a modification (Homandberg et al., 1985) of a previously reported method (March et al., 1974). Plasma fibronectin (PFn) was coupled to cyanogen bromide activated Sepharose 4B-CL in the presence of N-acetylated heparin as described previously (Ogamo et al., 1985).

Preparation and Analysis of PFn and PFn Proteolytic Fragments. PFn was isolated from outdated human plasma by using gelatin–Sepharose chromatography with freshly deionized 3 M urea–TBS elution (Engvall & Ruoslahti, 1977). For certain experiments, PFn was eluted from gelatin–Sepharose with 0.02 M sodium citrate pH 6 buffer (Miekka et al., 1982). PFn concentration was determined from its absorbance at 280 nm by using its reported extinction coefficient [$A^{1\%}_{1cm}$ = 12.8] (Mosesson & Umfleet, 1970). The pooled PFn-containing fractions from gelatin–Sepharose were dialyzed against Tris-buffered saline (TBS) containing protease inhibitors and sodium azide (50 mM Tris-HCl, 130 mM NaCl, 1 unit/mL Trasylol, 1 mM EDTA, 0.1 mM PMSF, and 0.02% sodium azide, pH 7.4), stored at room temperature and used within 4 days of isolation. Upon SDS–PAGE (Laemmli, 1970) of reduced specimens on 5% polyacrylamide gels, PFn migrated as a closely spaced doublet at 220–225 kDa. SDS–PAGE under nonreducing conditions confirmed that greater than 90% of the total protein migrated as the 450-kDa dimer with the majority of the remainder migrating in the position of the 235-kDa PFn component (Chen et al., 1977).

The 190/170-, 150/136-, and 29-kDa PFn fragments were isolated from a thrombin digest in the following way. PFn was incubated with thrombin (5 units/mL for 5 days) and then

applied to a gelatin–Sepharose column (Homandberg & Erickson, 1986). The nonadherent protein fractions, containing primarily the 150/136- and 29-kDa PFn fragments, were concentrated by dialysis vs solid PEG, dialyzed against TBS, reapplied to an Ultrogel AcA44 column (1 × 90 cm), and developed in TBS at a flow rate of 15 mL/h. The 150/136- and 29-kDa PFn fragments eluted with K_{av}s of 0.10 and 0.43, respectively. The 3 M urea–TBS elution from gelatin–Sepharose consisted mainly of the 190/170-kDa fragment (>95%). An occasional 45 kDa contaminant was separated from the 190/170-kDa fragment, when present, by gel sieving on Ultrogel AcA44.

An amino-terminal 72-kDa fragment was isolated by gelatin–Sepharose chromatography of a 4-h cathepsin D digest as described previously (Balian et al. 1979). The carboxyl-terminal Hep II 33-kDa fragment was isolated from a 20-h cathepsin D digest. Briefly, the material nonadherent to gelatin–Sepharose was pooled, dialyzed against 20 mM Tris and 50 mM NaCl, pH 7.4, and applied in this buffer to heparin–Sepharose. After the column was extensively washed with 20 mM Tris and 50 mM NaCl pH 7.4 buffer, bound peptides were eluted with a linear salt gradient (50–700 mM NaCl). The protein peak eluting at 375 mM NaCl was pooled, concentrated by dialysis vs solid PEG, and then dialyzed against TBS. Gel sieving this material on Ultrogel AcA44 yielded a 33-kDa preparation free of other contaminating peptides. The 100-kDa Hep III fragment was a gift from Dr. G. Homandberg (Abbott Laboratories, Abbott Park, IL) and was isolated as described previously (Homandberg & Erickson, 1986). The molecular weight of this fragment (estimated by SDS–PAGE) has previously been reported to be 120000 (Homandberg & Erickson, 1986).

The 190/170-kDa thrombin fragment was analyzed on 5% Laemmli gels, whereas the other PFn fragments were analyzed on 5–20% gradient gels after reduction with 5% β-mercaptoethanol. The Bradford protein dye binding assay with PFn employed as standard was used to determine the concentration of PFn fragments (Bradford, 1976).

Analytical Ultracentrifugation. Sedimentation velocity measurements in TBS were performed at 20 °C in a Beckman Model E analytical ultracentrifuge equipped with a photoelectric ultraviolet scanner. Centrifugation runs were at 56000 rpm. Samples were adjusted to give an optical density of 0.60 at 280 nm.

Amino Acid Sequencing. Peptides were dialyzed extensively against 0.10% acetic acid and then subjected to automated sequencing on a Model 477A Applied Biosystems pulsed liquid phase sequencer with a Model 120A online PTH amino acid analyzer at the Protein–Nucleic Acid Shared Facility at the Medical College of Wisconsin, Milwaukee, WI.

Preparation and Analysis of Fl-Heparin. Heparin was fluorescein-labeled as described previously (Ogamo et al., 1982). Labeled material (30 mg), equivalent to 3 times the maximum binding capacity of the PFn affinity resin, was applied to a 250-mL PFn–Sepharose column (2.2 mg of PFn/mL) that had been equilibrated in 50 mM Tris and 50 mM NaCl pH 7.4 buffer at 4 °C. After the column was washed with 2 L of the equilibration buffer, the bound Fl-heparin was eluted with 50 mM Tris and 500 mM NaCl pH 7.4 buffer, dialyzed against distilled water (in 6000–8000 M_r cutoff tubing), lyophilized, and stored at −20 °C.

The extent of fluorescein labeling was determined from Fl-heparin absorbance at 490 nm (ϵ = 89400 M^{-1} cm^{-1} at pH 10) (Ogamo et al., 1982). The calculation of the number of moles of label incorporated per mole of heparin assumed heparin and disaccharide molecular weights of 13800 and 400, respectively. Heparin was quantitated by using Azure A employing its heparin-induced absorbance decrease at 635 nm (Lam et al., 1976). Ultrogel AcA44 gel chromatography was used to estimate the molecular weight distribution of the Fl-heparin preparations (Johnson & Mulloy, 1976).

Fluorescence Polarization Binding Measurements. Fluorescence polarization measurements were made in a Perkin-Elmer Model 3000 spectrofluorometer equipped with Polaroid film polarizers. Fl-heparin emission at 525 nm was measured by using an excitation wavelength of 480 nm. Polarization (P) was determined by the L-format (i.e. single-channel) method (Lakowicz, 1983). Before analysis, protease inhibitors and sodium azide were removed from protein samples by dialysis.

Two equivalent methods were used to construct fluorescence polarization titration curves. Fluorescence polarization was either monitored at a fixed concentration of Fl-heparin (~14 nM) and varying concentratins of PFn/PFn fragment (0–4 μM) (method A) or at a fixed concentration of PFn/PFn fragment (~0.1 mg/mL) and varying concentrations of Fl-heparin (1–4000 nM) (method B).

Titrations were done in quadruplicate in 1-cm quartz cuvettes at 25 °C unless stated otherwise. The heparin-bound fraction, f_b, was calculated from

$$f_b = \frac{P - P_{min}}{P_{max} - P_{min}} \quad (1)$$

where P is the polarization at a given condition and P_{min} and P_{max} are the polarizations of free and totally bound Fl-heparin, respectively. Application of eq 1 assumes that the fluorescence quantum yields of Fl-heparin in its free and bound states are equal. Since the PFn-induced quenching of Fl-heparin fluorescence was small (14%), this assumption appears reasonable. P_{max} was estimated from polarization measurements on a Fl-heparin sample to which a 500-fold molar excess of PFn had been added.

The bound heparin concentration, $[heparin]_b$, was calculated at each titration point by using

$$[heparin]_b = f_b[heparin]_t \quad (2)$$

where f_b is defined by eq 1 and $[heparin]_t$ is the total heparin concentration. Scatchard plots were constructed by either plotting $[PFn\ derivative]_b$ vs $[PFn\ derivative]_b/[PFn\ derivative]_f$ (method A) or $[heparin]_b$ vs $[heparin]_b/[heparin]_f$ (method B), where the subscripts f and b denote the free and bound ligand concentration, respectively.

The bound concentration of PFn/PFn derivative, $[PFn\ derivative]_b$, was determined via its stoichiometric relationship to $[heparin]_b$

$$[PFn\ derivative]_b = f_b[heparin]_t/n \quad (3)$$

where f_b and $[heparin]_t$ are defined as above and n is the number of heparin-binding sites per molecule. The analyses for PFn and its proteolytic fragments assumed n values of 2 and 1, respectively. The Scatchard data were analyzed by using Munson's nonlinear least-squares curve-fitting program, LIGAND (Munson & Rodbard, 1980).

Results

We employed fluorescence polarization to quantitate fluid-phase heparin binding to PFn and its proteolytic fragments. Unfractionated Fl-heparin preparations undergo only small changes in fluorescence polarization in the presence of PFn (Bentley et al., 1985a) as a consequence of heparin po-

Table I: Fluorescence Polarization Derived Heparin Affinities of PFn and Its Proteolytic Fragments

derivative	conditions[a]	method[b]	dissociation constant, K_d (nM) Fl-heparin prepn 1	Fl-heparin prepn 2
PFn	TBS	A	217 ± 12	ND
PFn	TBS	B	0.73 ± 0.69, 118 ± 8	0.90 ± 0.75, 173 ± 28
PFn	PBS	B	ND	0.29 ± 0.40, 452 ± 46
PFn	PBS, 5 °C	B	ND	2.9 ± 1.4, 356 ± 96
190/170 kDa	TBS	A	492 ± 22	884 ± 61
190/170 kDa	TBS	B	ND	5.6 ± 6.7, 446 ± 119
150/136 kDa	TBS	A	278 ± 130	ND
33 kDa	TBS	A	2857 ± 286	ND
33 kDa	TBS	B	ND	893 ± 20
72 kDa	TBS	A	~30000	ND
29 kDa	TBS	A	~30,000	ND
100 kDa	TBS	A	>30000	ND

[a] Titrations performed either in 50 mM Tris-HCl and 130 mM NaCl, pH 7.4 (TBS), or 33 mM sodium phosphate and 100 mM NaCl, pH 7.4 (PBS). Measurement at 25 °C unless otherwise stated. [b] Titration method. Method A: Fl-heparin fixed; PFn/PFn fragment varied. Method B: PFn/PFn fragment fixed; Fl-heparin varied.

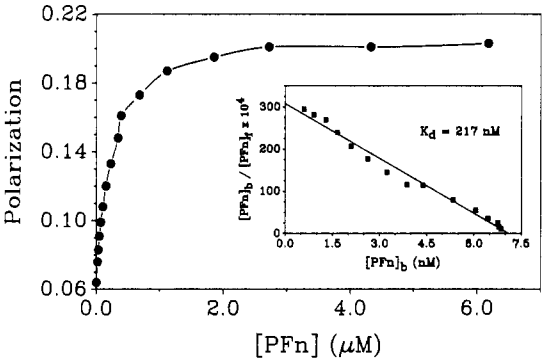

FIGURE 2: Fluorescence polarization titration curve obtained by incubating a fixed amount of Fl-heparin (14 nM, preparation 1) with varying concentrations of PFn in TBS at 25 °C (method A under Materials and Methods). (Inset) Scatchard plot derived from the titration data. At P_{max} all of the Fl-heparin present is assumed to be bound. $[PFn]_b$ was calculated by assuming a 2:1 heparin:PFn stoichiometry and a PFn molecular weight of 450000.

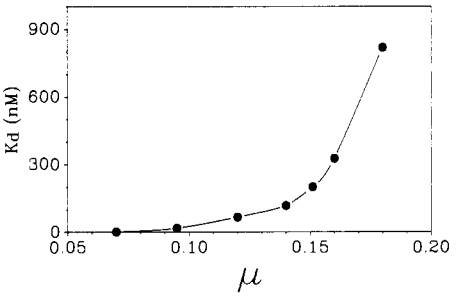

FIGURE 3: Effect of ionic strength on the K_d of the heparin–PFn interaction. Method A under Materials and Methods was used to determine K_d. Increasing salt was added to 50 mM Tris-HCl pH 7.4 buffer at 25 °C.

Table II: $s_{20,w}$ Values for the PFn Fragments Employed in This Study

derivative	$s_{20,w}$	ref
PFn	11.8	Mosesson et al. (1975)
190/170 kDa	6.0	this study
150/136 kDa	4.9	this study
100 kDa	5.4	Homandberg and Erickson (1986)
72 kDa	3.9	Odermatt et al. (1982)
33 kDa	2.0	this study
29 kDa	2.2[a]	Odermatt et al. (1982)

[a] Fragment studied was obtained by plasmin degradation of the 72-kDa fragment. This peptide is structurally homologous to the thrombin-derived 29-kDa fragment investigated here.

lydispersity with respect to size and charge (Laurent et al., 1978; Hurst et al., 1979, 1983). In order to increase the sensitivity of these analyses, we first enriched our Fl-heparin preparations in their "PFn-bindable" fraction by the use of PFn–Sepharose affinity chromatography.

Two different preparations of Fl-heparin were used in this study. The first contained 1.9 mol of fluorescein/mol of heparin and exhibited P_{min} and P_{max} values near 0.06 and 0.21, respectively. The second contained 0.70 mol of fluorescein/mol of heparin and exhibited P_{min} and P_{max} values of 0.07 and 0.33, respectively. The lower P_{max} exhibited by the first Fl-heparin preparation reflects averaging of the inequivalent rotational diffusion rates of multiple label molecules. The molecular weight distributions of the two Fl-heparin preparations were similar ($\bar{M}_r$ 13800; gel sieving data not shown).

Heparin Binding to PFn. When a fixed amount of Fl-heparin (~14 nM) was incubated in TBS (μ = 0.15) with increasing concentrations of PFn, an increase in fluorescence polarization was observed (Figure 2). This polarization change reflects the decreased rotational diffusion rate of Fl-heparin upon binding to PFn. Scatchard analysis (inset) yielded a linear plot indicative of a single class of heparin-binding sites (K_d = 217 nM). Identical results were obtained with a PFn preparation isolated in the absence of urea. This indicates that brief exposure of PFn to 3 M urea during isolation does not modify its intrinsic heparin affinity. There was a progressive and marked rise in K_d (Figure 3) as the buffer ionic strength was increased above 0.15. Heparin–PFn complexation was weak above μ = 0.20 (K_d > 1000 nM).

Comparison of the relative affinities of PFn for the two Fl-heparin preparations employed indicated that the second preparation yielded somewhat higher dissociation constants (Table I). This variation probably reflects subtle differences in the degree of sulfation of the two Fl-heparin preparations. However, the relative affinities of PFn and its proteolytic fragments for each Fl-heparin preparation were essentially the same (Table I).

Heparin Binding to PFn Fragments. In order to assess the respective participation of the various reported PFn heparin-binding domains in its interaction with heparin, we evaluated the Fl-heparin-binding affinities of a series of PFn fragments. The structural properties of the fragments under present study are illustrated in Figure 1. We assumed for purposes of our data analysis that each fragment studied was monomeric in solution. This assumption is supported by their relatively slow $S_{20,w}$ values, compared to that of dimeric PFn, reported in the literature or determined in this study (Table II).

Comparison of the titration curves of PFn and the 190/170-kDa fragment (Figure 4A) obtained at constant heparin concentration (method A) indicated that both bound Fl-he-

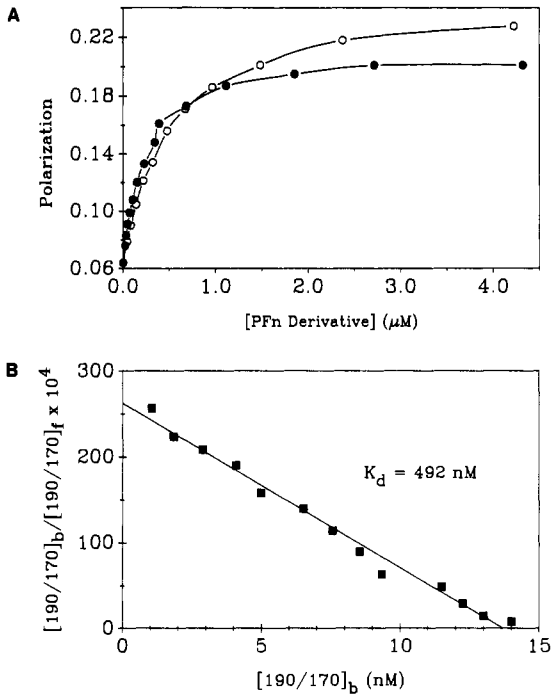

FIGURE 4: (A) Comparison of the titration curves of PFn (closed circles) and the 190/170-kDa fragment (open circles). Conditions same as Figure 2. P_{max} approached 0.24 for the 190/170-kDa PFn fragment. (B) Scatchard plot derived from the 190/170-kDa titration data. Calculation of [190/170 kDa]$_b$ assumed a 1:1 heparin:190/170 kDa stoichiometry.

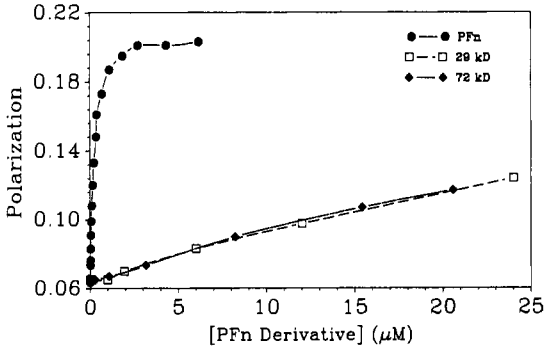

FIGURE 5: Comparison of the titration curve of PFn (closed circles) with that of the amino-terminal 29-kDa (open squares) and 72-kDa (closed diamonds) PFn fragments containing Hep I. Conditions same as in Figure 2.

parin with similar affinity. Scatchard analysis of the 190/170-kDa fragment titration data (Figure 4B) yielded a linear plot indicative of a single major class of heparin-binding sites with a K_d of 492 nM. The 150/136-kDa fragment yielded similar results (Table I). These observations indicate that large carboxyl-terminal PFn fragments, containing both Hep II and Hep III domains, retain a heparin-binding affinity close to that of the native dimeric PFn molecule. The Fl-heparin affinity of the carboxyl-terminal 33-kDa fragment containing Hep II was still appreciable, although it was 2–5-fold lower than that of the 190/170-kDa fragment (Table I).

In contrast to the relatively high heparin-binding affinity of the fragments containing Hep II, the amino-terminal 72- and 29-kDa Hep I fragments exhibited low heparin affinity (Figure 5). Although rigorous Scatchard analysis of the 29- and 72-kDa fragment titration data was not possible due to the weak binding, we estimate a K_d near 30 000 nM for these fragments containing Hep I. Heparin binding to the 100-kDa fragment containing Hep III was barely detectable at physiologic buffer conditions (K_d > 30 000 nM; Table I) and was

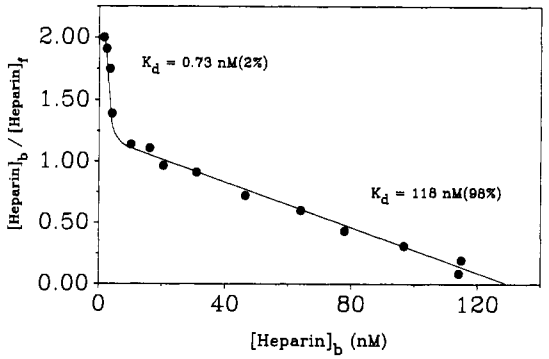

FIGURE 6: Scatchard plot derived from a titration in which increasing amounts of Fl-heparin were added to a TBS solution containing a fixed amount of PFn (0.08 mg/mL) at 25 °C. Method B was employed. The x intercept of this plot corresponds to approximately 0.70 heparin-binding site/mol of PFn.

even weak at low ionic strength (μ = 0.07, K_d = 2000 nM; data not shown).

Evidence for a Second High-Affinity Heparin-Binding Activity. Previous experiments using fixed heparin concentrations and varying concentrations of PFn (method A) in TBS indicated a single class of high-affinity heparin-binding sites. However, we detected an additional class of high-affinity heparin-binding sites in TBS (Figure 6) when a fixed concentration of PFn was incubated with increasing concentrations of Fl-heparin (method B). This latter activity accounts for only a small fraction (<2%) of the total heparin-binding sites on PFn. Similar results were obtained by using method B for PFn in phosphate buffer (μ = 0.17) at both 5 and 25 °C (Table I). The high-affinity component was also present in the 190/170-kDa PFn fragment but was not detectable in the 33-kDa Hep II fragment under any conditions (Table I). Scatchard analysis of the 150/136-kDa PFn fragment binding data also suggested the presence of two components (data not shown), but the limited availability of the material precluded precise determination of dissociation constants. Gel-sieved Fl-heparin, which lacked a minor population of larger glycosaminoglycans (M_r > 18 000) that were usually present in standard Fl-heparin preparations, still yielded a PFn titration curve identical with that obtained with the unchromatographed starting material (data not shown).

Discussion

The information obtained in this study allows us to assess the respective participation of the three reported PFn heparin-binding domains under physiological buffer conditions. PFn fragments containing either the amino-terminal Hep I or carboxyl-terminal Hep II domains bind to heparin–Sepharose columns under conditions of physiological pH and ionic strength (Smith & Furcht, 1982; Gold et al., 1983; Hayashi & Yamada, 1983). However, the affinity of PFn fragments containing Hep II for heparin–Sepharose is significantly higher than that of fragments containing Hep I (Gold et al., 1983). Consistent with this, we found that Hep I fragments bound heparin very weakly with a K_d near 30 000 nM, whereas under the same conditions the large carboxyl-terminal PFn fragments containing Hep II (190/170 and 150/136 kDa) bound heparin nearly as well as did native PFn (K_d = 278–492 nM). It is evident that heparin–Sepharose affinity chromatography alone is of limited utility in the quantitative assessment of heparin-binding potential, and from our results we can conclude that the Hep I domain does not participate significantly in the fluid-phase interaction between heparin and PFn.

Table III: K_ds for the Heparin–Fn Interaction in the Literature

study	assay method	conditions[a]	Fn source	K_d[b] (nM)
Yamada et al. (1980)	solid phase	A	chick fibroblast	4, 120
Tarsio et al. (1987)	solid phase	A	plasma	20–30, 200
Bentley et al. (1985a)	FP	B	plasma	35, 1000
present	FP	C	plasma	0.73–0.90 (<2%), 118–173 (>98%)
present	FP	D	plasma	0.29 (1%), 452 (99%)

[a] Experimental conditions: (A) 23 °C, 10 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 1.5 mM KH_2PO_4, 18.1 mM Na_2HPO_4 (pH 7.4); (B) 4 °C, 50 mM sodium phosphate, 100 mM NaCl (pH 7.2); (C) 25 °C, in 50 mM Tris-HCl, 130 mM NaCl (pH 7.4); (D) 25 °C, 33 mM sodium phosphate, 100 mM NaCl (pH 7.4). [b] All data shown in the table were derived from titrations in which the Fn concentration was fixed while the heparin concentration was varied.

We observed that PFn and the large fragments containing Hep II bound Fl-heparin 60–150-fold more tightly than PFn fragments that contained either the Hep I or Hep III domain alone. From this observation, we conclude that PFn contains only one major heparin-binding site per subunit, Hep II. This conclusion is consistent with the previous finding of a single heparin-binding site in each subunit of chick embryo fibroblast Fn (Yamada et al., 1980). Although we have assumed in our analysis of the PFn binding data that each dimeric molecule contains one heparin-binding site per subunit, we cannot exclude a model in which Hep II domains on adjacent or contiguous subunits act in concert to form a single heparin-binding site on the dimeric molecule. Our findings exclude models in which the Hep I or Hep III domains function independently (Gold et al., 1983; Hayashi & Yamada, 1982).

Gold and co-workers (1983) have localized a low-affinity Hep III domain to a 30-kDa region toward the center of the PFn subunit (Figure 1). In agreement with this previous finding, we observed that Fl-heparin binding to the 100-kDa fragment containing Hep III was barely detectable under physiologic buffer conditions (K_d > 30 000 nM). However, the large carboxyl-terminal 190/170-kDa PFn fragment, containing both Hep II and Hep III, exhibited 2–5-fold higher heparin affinity than that of the isolated Hep II domain (33-kDa fragment). Therefore, the presence of Hep III in PFn or PFn fragments containing Hep II may enhance Hep II affinity by some cooperative mechanism.

Recently, Thompson and co-workers (1986) used fluorescence polarization to investigate ganglioside binding to PFn. They localized this activity to a 31-kDa PFn fragment that they believed to be derived from the amino-terminal region of PFn. However, this fragment also bound heparin with high affinity, as evaluated by fluorescence polarization. On the basis of the latter observation, we believe their assignment of both ganglioside and heparin-binding activity to the amino-terminal region of PFn is probably not correct, since tight heparin-binding activity is attributable only to the carboxyl-terminal Hep II domain.

We have not elucidated the origin of the high-affinity heparin-binding sites that we and others (Yamada et al., 1980; Bentley et al., 1985a; Tarsio et al., 1987) have observed. This activity is present in PFn and the 190/170- and 150/136-kDa fragments. We believe this activity arises from multivalent binding of heparin molecules to PFn or to the larger fragments containing Hep II. This may occur either by intramolecular heparin binding to multiple sites on a single PFn subunit or by intermolecular binding in which a single heparin molecule bridges sites on different molecules.

Comparison of our PFn binding data with those already reported in the literature (Table III) indicates that the heparin-binding characteristics of fluid-phase PFn, solid-phase PFn, and insoluble chick fibroblast Fn are quite similar. This suggests that each binds heparin in the same way, even though there are differences in their physical form (i.e., soluble dimers vs insoluble dimers and multimers). The previous fluorescence polarization study by Bentley et al. (1985a) reported somewhat higher K_ds than we did for fluid-phase PFn, probably reflecting the higher ionic strength (μ = 0.20) buffer employed by these investigators (Table III).

Leivo et al. (1986) have investigated the interaction of PFn and several cathepsin G derived PFn fragments with an endodermal cell matrix containing heparan sulfate proteoglycan as its major (>90%) glycosaminoglycan component. They found that PFn and a 140/120-kDa fragment containing Hep II bound to this cell matrix, while fragments containing either the isolated Hep I (30 kDa) or collagen-binding (40 kDa) domains did not. Taken together with our present findings, these results apparently imply a role for Hep II in the attachment of PFn to extracellular matrix of the endodermal PSY-2 cell line.

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Combining Thermostable Mutations Increases the Stability of λ Repressor†

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ABSTRACT: We have combined three mutations previously shown to stabilize λ repressor against thermal denaturation. Two of these mutations are in helix 3, where Gly-46 and Gly-48 have been replaced by alanines [Hecht, M. H., et al. (1986) *Proteins: Struct., Funct., Genet. 1*, 43–46]. The other mutation, which replaces Tyr-88 with cysteine, allows the protein to form an intersubunit disulfide bond [Sauer, R. T., et al. (1986) *Biochemistry 25*, 5992–5998]. Calorimetric measurements show that the two alanine substitutions stabilize repressor by about 8 °C, that the disulfide bond stabilizes repressor by about 8 °C, and that the triple mutant is 16 °C more stable than wild-type repressor.

Do we understand proteins well enough to systematically design thermostable variants? Can changes be combined to generate "hyperstable" variants? Many interactions are important for stabilizing proteins—hydrophobic interactions, van der Waals contacts, hydrogen bonds, and salt bridges. Since each interaction might contribute only a few kilocalories per mole to the net stability of a protein, we expect that a number of changes must be combined to generate significant increases in the thermostability. To test this approach, we have combined several existing mutations in the amino-terminal domain of λ repressor (residues 1–92) and measured the thermostability of the new protein. In a previous study, two glycines in helix 3 of repressor were replaced with alanines in order to stabilize that helix (Hecht et al., 1986). Each Gly → Ala substitution increased the thermostability of intact repressor by 3–5 °C, while the double mutant increased the thermost-

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