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Interaction of heparin with fibronectin and isolated fibronectin domains

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Fluorescence polarization, gel exclusion chromatography and affinity chromatography were used to characterize the interaction of heparins of different size with human plasma fibronectin (Fn) and several of its isolated domains. The fluid-phase interaction of Fn with heparin was dominated by the 30 kDa and 40 kDa Hep-2 domains located near the *C*-terminal ends of the A and B chains respectively. The 30 kDa Hep-2A domain from the heavy chain was indistinguishable from the 40 kDa Hep-2B domain in this respect; the presence of an additional type III homology unit in the latter had no effect on the binding. Evidence was provided that each Hep-2 domain has two binding sites for heparin. The *N*-terminal Hep-1 domain reacted weakly in fluid phase even though it binds strongly to immobilized heparin. Fn and Hep-2 fragments were rather undiscriminating in their reaction with fluoresceinamine-labelled heparins of different sizes. However, oligosaccharides smaller than the tetradecasaccharide (14-mer) bound Fn with a 5–10-fold lower affinity. These results suggest that the Hep-2 domains of Fn are able to recognize a broad spectrum of oligosaccharides that presumably vary significantly with respect to the amount and spatial distribution of charge.

INTRODUCTION

Fibronectin (Fn) is a 500 kDa glycoprotein found in plasma and other body fluids and on the surfaces of numerous types of cells [see reviews by Mosesson & Amrani (1980), Mosher (1980), Yamada (1983), Hynes (1985) and Ruoslahti (1988)]. It is composed of two nearly identical polypeptide chains, each of which contains numerous domains that can be isolated from proteolytic digests with retention of specific macromolecular recognition properties. Fn binds collagen and glycosaminoglycans, and mediates the attachment of various cells to surfaces containing these substances. This attachment generally involves specific cell-surface adhesion receptors that recognize a tripeptide sequence, Arg-Gly-Asp (RGD), located within the cell-attachment domain of Fn. However, there is growing evidence that glycosaminoglycan-binding domains of Fn may also participate in the adhesion and/or spreading process, either in concert with or independent of the RGD mechanism, through recognition of proteoglycans located on the surfaces of some cells (Izzard *et al.*, 1986; Woods *et al.*, 1986; Lewandowska *et al.*, 1987; Rogers *et al.*, 1987; McCarthy *et al.*, 1988; LeBaron *et al.*, 1988; Hall *et al.*, 1988; Saunders & Bernfield, 1988). Heparin-like molecules have been shown to inhibit the attachment of certain cells to Fn-coated surfaces (Klebe & Mock, 1982), and the glycosaminoglycan-binding domains of Fn have been implicated in several important processes such as endothelial-cell growth (Homandberg *et al.*, 1985, 1986), neurite outgrowth (Rogers *et al.*, 1987) and tumour-cell invasion (McCarthy *et al.*, 1988).

At least three different regions of the Fn molecule have been implicated in its binding to heparin (Gold *et al.*, 1983). Their locations within the polypeptide chains are illustrated in Fig. 1(*a*). The first is located in a 25 kDa *N*-terminal domain, Hep-1, which is rapidly released when Fn is digested with a variety of proteinases. It has a net positive charge (pI 8.2–8.4) and is rich in disulphide bonds, being comprised of five repeats of the type I homology units, or fingers (Skorstengaard *et al.*, 1986). The second domain, Hep-2, is located near the opposite end of the molecule, adjacent to the variable region, V, which accounts for the heterogeneity between the A and B chains of plasma Fn. It also bears a net positive charge (pI 8.7–9.2) and is composed of three consecutive type III homology units, which are devoid of disulphide bonds. This domain is sometimes referred to as the high-affinity heparin-binding domain, because it requires the highest concentration of NaCl to be eluted from heparin–Sepharose. It is easily isolated as a mixture of 30 kDa and 40 kDa fragments from proteolytic digests of plasma Fn; the absence of the V region from the B chain eliminates a cleavage site and results in the inclusion of an extra type III homology unit in the fragment derived from that chain (see Fig. 1). An additional site of weak binding to heparin is found adjacent to the gelatin-binding domain on the *C*-terminal side (Calaycay *et al.*, 1985). It is included in larger gelatin-binding fragments obtained from partial digests. Binding of heparin to this site is relatively weak, occurring only at low (sub-physiological) ionic strength. A fourth site, first identified as a DNA-binding site, is also reported to bind heparin at low ionic strength (Pande *et al.*, 1985).

In contrast with the substantial progress in localizing heparin-binding sites within the Fn chains, our understanding of the binding mechanisms and equilibrium constants is much less developed, as is our understanding of the features of the heparin molecule that are important for recognition by Fn. Yamada *et al.* (1980), using a filter binding assay, observed heterogeneous binding with a dissociation constant, K_d, of approx. 0.01–0.1 μM in phosphate-buffered saline at 23 °C. Bentley *et al.* (1985) utilized the change in fluorescence polarization of fluoresceinamine-labelled heparin (FA–heparin) to arrive at a similar range of values under the same conditions at 2 °C. Evington *et al.* (1985) utilized similar techniques and conditions to obtain a substantially higher K_d of 2 μM. Benecky *et al.* (1988) also utilized this method to provide information about the affinities of heparin for the various fragments of Fn. In the present study, we systematically examine the effects of pH, temperature and ionic

Abbreviations used: Fn, fibronectin; FA–heparin, fluoresceinamine-labelled heparin.

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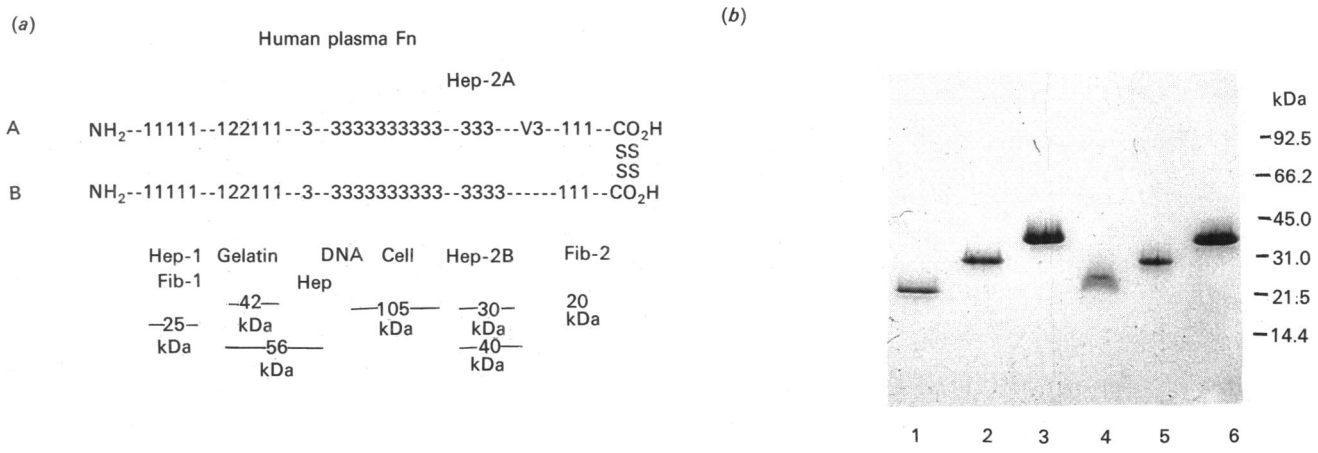

Fig. 1. Structure and purity of Fn and its fragments

(*a*) Schematic representation of the structure of human plasma Fn. The relative location of the fragments in the polypeptide chain(s) is illustrated in the schematic, where the numerals 1, 2, and 3 represent the three types of internal homologous repeat structures. V represents a variable insert whose presence in the A chain introduces a proteinase-sensitive site not present in the B chain, accounting for the absence of the additional type 3 homology unit in the 30 kDa Hep-2A domain (Skorstengaard *et al.*, 1986). (*b*) PAGE of fragments used in this study. Purified fragments were electrophoresed in the presence of SDS without (lanes 1–3) and with (lanes 4–6) prior reduction. Lanes 1 and 4, *N*-terminal 25 kDa Hep-1 fragment; lanes 2 and 5, high-affinity 30 kDa Hep-2A fragment; lanes 3 and 6, high-affinity 40 kDa Hep-2B fragment. Positions of molecular-mass markers are indicated on the right.

strength on the dissociation constant for fluid-phase binding of Fn to FA–heparin. In addition, we provide the first measure of dissociation constants for oligomeric fragments of heparin to Fn. Finally, we present evidence that each Hep-2 domain has two binding sites for heparin.

MATERIALS AND METHODS

Preparation of Fn and isolated Fn domain fragments

Fn was purified according to method B of Miekka *et al.* (1982). Fragments were generated with thermolysin and purified as described previously (Zardi *et al.*, 1985; Borsi *et al.*, 1986). The 56 kDa fragment of Fn was isolated by affinity fractionation on heparin–Sepharose (Isaacs *et al.*, 1989). The Hep-1, Hep-2A and Hep-2B fragments and the 56 kDa fragment bound to heparin–Sepharose quantitatively when applied in 0.02 M-Tris, with no significant evidence of non-binding material. The concentrations of NaCl required for peak elution of Hep-1, Hep-2A and Hep-2B fragments and the 56 kDa fragment were 0.50 M, 0.63 M, 0.57 M and 0.18 M respectively. SDS/PAGE of the purified Hep-1, Hep-2A and Hep-2B fragments is shown in Fig. 1, which also shows the location of these and other fragments within the polypeptide chain(s). Additional evidence of purity is provided by the exclusion-chromatography profiles in Fig. 4. Protein concentrations were determined from the absorbance at 280 nm by using the following absorption coefficients ($A^{1\%}_{1\,cm}$): Fn, 12.8; Hep-1, 17.9; Hep-2A and Hep-2B, 10.5 (Ingham *et al.*, 1984). The identity of the fragments was confirmed by *N*-terminal sequence analysis. The Hep-1 fragment was blocked at the *N*-terminus, and as expected exhibited the characteristic increase in molecular mass on reduction (Borsi *et al.*, 1986).

Preparation of labelled heparin and oligosaccharides

Pig mucosal heparin (sodium salt) (Sigma Chemical Co., St. Louis, MO, U.S.A.) was labelled with fluoresceinamine isomer II (Sigma Chemical Co.) by the method of Ogama *et al.* (1982). The product was fractionated on a 1.4 cm × 90 cm column of Sephadex G-100 (Pharmacia, Piscataway, NJ, U.S.A.) in 0.02 M-Tris/HCl buffer, pH 7.4, containing 0.5 M-NaCl, the absorbance at 495 nm being monitored. Values of $K_{av.}$ were calculated from the formula:

$$K_{av.} = (V_e - V_0)/(V_t - V_0)$$

where V_e is the peak elution volume of the sample, V_0 is the elution volume of a totally excluded substance (thyroglobulin) and V_t is the bed volume of the column. The eluted material was divided into seven pools and analysed for fluorescein content (ϵ 72000 $M^{-1} \cdot cm^{-1}$ at 495 nm; Chen, 1969) and uronic acid content (Bitter & Muir, 1962) to determine the degree of labelling.

Fluorescent labelled heparin was also prepared by a procedure described by Glabe *et al.* (1983), in which the fluoresceinamine is allowed to react with CNBr-activated heparin. The resulting product, unfractionated, produced a response to Fn that was very similar to the FA–heparin prepared by the Ogama method, with K_d 1.5 ± 0.5 μM. This provides additional evidence that the probe does not significantly influence the interaction, since the two labelling methods involve different chemical linkages to heparin.

To prepare oligomers of heparin, 1 g of pig mucosal heparin was dissolved in 20 ml of cold 0.2 M-citric acid and the pH adjusted to 1.5 with conc. H_2SO_4. $NaNO_2$ was added to 0.05 M. After 2 min at 0 °C, the reaction was quenched with ammonium sulphamate (0.075 M). The reaction mixture was precipitated with cold 80% (v/v) ethanol and then centrifuged at 4 °C, and the pellet was dissolved in water. The oligosaccharides were labelled with fluoresceinamine as above and dialysed against water with a 1000 Da-cut-off membrane (Spectrum Inc., Los Angeles, CA, U.S.A.) and fractionated by gel filtration on a polyacrylamide P-10 (Bio-Rad Laboratories, Richmond, CA, U.S.A.) column (1.5 cm × 100 cm) at 6 ml/h in 0.5 M-ammonium bicarbonate buffer, pH 8.5, the fluorescein absorbance at 495 nm being monitored. The fractions were selected from peaks ranging from tetrasaccharide to tetradecasaccharide (Oosta *et al.*, 1981). The disaccharides and most of the tetrasaccharides were removed by the previous dialysis step.

Gel electrophoresis and chromatography

SDS/PAGE was performed with the Pharmacia Phast gel

system with 8–25% -acrylamide-gradient pre-cast gels. Samples containing 0.5–1.0 mg of protein/ml in Tris-buffered saline (0.02 M-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl and 0.02% NaN_3) and either 1% SDS or 1% SDS plus 3% thioglycerol were heated at 100 °C for 2 min before application to the gel along with molecular-mass standards obtained from Bio-Rad Laboratories. After electrophoresis for 85 V·h, the gel was developed by staining with Coomassie Brilliant Blue R250.

Exclusion chromatography was performed on a Pharmacia f.p.l.c. system with a Superose-12 column (1.0 cm × 30 cm) at a flow rate of 1.0 ml/min. Elution was monitored by absorbance at 280 nm or fluorescence at 350 nm with a Shimadzu RF-530 detector. Affinity chromatography was also performed on the f.p.l.c. system. Unbleached heparin containing unsubstituted amino groups, Fn and its fragments were coupled to CNBr-activated Sepharose (Sigma Chemical Co.) in 0.2 M-sodium bicarbonate buffer, pH 8, containing 0.5 M-NaCl for 2–3 h at room temperature. Sites on the gel that had not reacted were blocked with ethanolamine. It was determined, by measurement of the absorbance of the supernatant at 280 nm after coupling, that 4.0 mg of Fn, 0.8 mg of Hep-1 fragment and 2.0 mg of Hep-2A fragment had bound per ml of packed gel. Affinity columns of approx. 1.5 ml volume were utilized in analytical experiments. The elution of FA–heparin was monitored by fluorescence at 525 nm with excitation at 485 nm.

Fluorescence anisotropy measurements

Fluorescence anisotropy measurements were made on an SLM 8000C fluorimeter in the T format with excitation and emission wavelengths of 493 nm and 524 nm respectively (Isaacs *et al.*, 1989). The temperature was controlled by a circulating water bath. Titrations of FA–heparins were performed in Tris-buffered saline, unless otherwise indicated, by addition of small amounts of a stock solution of the titrant. For pH titrations, pH was measured after each addition of 1 M-HCl before the anisotropy measurement. Each anisotropy value is the average of 10–15 independent determinations. The change in anisotropy, ΔA, as a function of titrant concentration was fitted to a single class of equivalent binding sites on Fn (or fragment) by using eqn. (1):

$$\Delta A = \Delta A_{max.}\left[\frac{K_d + n[Fn] + [H]}{2[H]} - \sqrt{\left(\frac{K_d + n[Fn] + [H]}{2[H]}\right)^2 - \frac{n[Fn]}{[H]}}\right] \quad (1)$$

where [Fn] and [H] are the total concentrations of Fn and heparin respectively and n is the number of heparin-binding sites on the Fn molecule. The adjustable parameter, $\Delta A_{max.}$, is the maximum anisotropy change that would be produced at saturating concentrations of Fn and K_d is the dissociation constant of the heparin–Fn complex. The derivation of this equation requires that $K_d = n[Fn_f][H_f]/[H\text{–}Fn]$, $[H] = [H_f] + [H\text{–}Fn]$ and $[H\text{–}Fn]/[H] = \Delta A/\Delta A_{max.}$, where $[Fn_f]$ and $[H_f]$ are the free concentrations of Fn and heparin respectively, [H] is (as above) the total concentration of heparin and [H–Fn] is the total concentration of heparin complexed with Fn, i.e. $[H_1\text{–}Fn] + 2[H_2\text{–}Fn] + 3[H_3\text{–}Fn] + \ldots n[H_n\text{–}Fn]$. This last assumption requires that the change in anisotropy of each heparin molecule resulting from its complex with Fn be independent of the number of heparin molecules that are already bound to the Fn. This is reasonable for small values of n, considering that the molecular masses of the heparin oligosaccharides range from about one-fiftieth to one-fivehundredth that of Fn.

RESULTS

Effect of heparin size on its binding to Fn

FA–heparin was fractionated according to size by exclusion chromatography on Sephadex G-100. Each of the seven pools displayed a relatively narrow elution profile when analysed by f.p.l.c. on a Superose-12 column. The $K_{av.}$ values were determined for the pools and the estimated molecular masses are summarized in Table 1. A sample from each pool was shown to bind quantitatively to Fn–Sepharose. When a linear gradient of NaCl was applied, all samples were eluted as single peaks at between 0.25 M- and 0.30 M-NaCl without apparent correlation between the size of the heparins and the concentration of salt required for their elution. This suggests that heparin size has little effect on the affinity for immobilized Fn.

To address this question further, several samples were titrated with Fn in Tris-buffered saline, the increase in anisotropy being used to monitor complex-formation in the fluid phase, as described in the Materials and methods section. The magnitude of the change in anisotropy decreased with decreasing size of the FA–heparin (Fig. 2*a*). Dissociation constants were obtained by fitting the data to eqn. (1), with a value of $n = 4$ for the stoichiometry of binding (see below). The resulting dissociation constants, summarized in the upper part of Table 1 (column 4), did not vary significantly with the size of the heparin over the range represented by the pools.

The effect of size was further investigated by titrating a series of oligomeric fractions of heparin that had been generated with HNO_2 and labelled with fluoresceinamine. The labelled oligosaccharides all bound to Fn–Sepharose quantitatively and were eluted with a linear gradient of NaCl in essentially the same range as the undegraded heparin (0.3–0.4 M). The labelled oligomers were titrated with Fn, the resulting changes in anisotropy being monitored as described above. The results are shown in Fig. 2(*b*), where the lines represent theoretical best fits of the data to eqn. (1), again with a value of $n = 4$. As shown for the 18–20-mer in Fig. 2(*c*), lower values of n were inadequate. The resulting K_d values are summarized in the lower half of Table 1. The larger oligomers had affinities similar to that of undegraded heparin; however, there was a significant decrease in affinity with oligomeric fractions smaller than the tetradecamer (14-mer), as evidenced by the approx. 5–10-fold increase in dissociation constant.

Unless noted otherwise, the experiments described below were done with labelled heparin pool 3.

Effect of solvent conditions

Measurements were made of the effect of pH on the binding of Fn to FA–heparin in the range from pH 8 to pH 6. Although the fluorescence intensity of the label was also quenched over this pH range, the residual intensity at pH 6 was sufficient for titrations to be conducted. The dissociation constants corresponding to the theoretical best fits of the data to eqn. (1) are given in Table 2. As shown, decreasing the pH from 8.0 to 6.0 caused a large increase in affinity, as manifested by the almost 50-fold decline in the dissociation constant.

The interaction between Fn and heparin was also sensitive to ionic strength. As shown in Table 2, K_d increased from 0.4 μM in 0.01 M-NaCl to 30 μM in 0.3 M-NaCl, a 70-fold change. Titration

Table 1. Summary of dissociation constants for binding of various size heparin fractions to Fn

Measurements were made at 25 °C in Tris-buffered saline, pH 7.4. K_d values were obtained by fitting titration data to eqn. (1) with $n = 4$. The molecular masses are approximate values based on $K_{av.}$ values and heparin standards.

Heparin fraction	$K_{av.}$	Molecular mass (Da)	K_d (μM)
Sephadex G-100 pool*			
1	0.06	30000	2.5
2	0.17	25000	–
3	0.26	20000	–
4	0.34	15000	2.5
5	0.47	10000	–
6	0.60	7000	–
7	0.87	5000	1.8
Oligosaccharides†			
18–20	0.16	6000	0.90
16	0.19	5200	1.1
14	0.24	4550	0.88
12	0.28	3900	5.0
10	0.35	3250	9.4
8	0.43	2600	11
6	0.52	1950	18

* Degree of labelling: 0.006–0.027 mol of dye/mol of disaccharide unit.

† Numbers represent nominal size of the oligomeric fractions in saccharide units. Degree of labelling: approx. 0.0013 mol of dye/mol of disaccharide unit.

of a solution of FA–heparin that was approximately half-saturated with Fn in Tris-buffered saline with $CaCl_2$, $MnCl_2$ or $MgCl_2$ had no significant effect on the anisotropy at concentrations up to 25 mM. A decrease in anisotropy (dissociation of the complex) was observed at higher concentrations, but the effect was no greater than that caused by NaCl at an equivalent ionic strength. Thus we saw no evidence for specific metal ion effects.

Binding of Fn to FA–heparin was remarkably insensitive to temperature. Titrations conducted at 10 °C and 37 °C produced dissociation constants that differed insignificantly from that obtained at 25 °C (results not shown).

Heparin binding to Fn fragments

Titrations of FA–heparin with several of its fragments are illustrated in Fig. 3(*a*). The 30 kDa Hep-2A and 40 kDa Hep-2B fragments caused a progressive increase in the fluorescence anisotropy, whereas the *N*-terminal 25 kDa Hep-1 fragment had a small effect. The 105 kDa cell-binding and 42 kDa gelatin-binding fragments produced no response at concentrations up to 10 μM (results not shown). A 56 kDa gelatin-binding fragment reported to contain a heparin-binding site in its *C*-terminal region (Calaycay *et al.*, 1985) also failed to respond under these conditions, as did albumin. The continuous curves in Fig. 3(*a*) represent theoretical best fits of the data to eqn. (1), with a stoichiometry of $n = 2$. As shown for the 14-mer in Fig. 3(*b*), the lower value of $n = 1$ was inadequate. The dissociation constants thus obtained are summarized in Table 2. The values for Hep-2A and Hep-2B fragments differ insignificantly from each other but are slightly higher than that of whole Fn. The response with the Hep-1 fragment was too small to yield a reliable K_d, but the results are consistent with a 20–30-fold lower affinity. In this regard, our measurements agree with those made by Benecky *et al.* (1988), that the fluid-phase interaction of Fn with FA–heparin can be accounted for primarily by the Hep-2 domains, with little contribution from the Hep-1 domain.

The increase in anisotropy of FA–heparin caused by the Hep-2 fragments could be readily reversed by excess unlabelled heparin. As shown in Fig. 3(*c*), 50% inhibition occurred near 10 μM, consistent with the K_d obtained by direct titration of the labelled heparin. Although the heterogeneity of the heparin precluded a more precise analysis of the inhibition data, it is clear that the presence of the label does not significantly influence the affinity for Hep-2 domains.

Several experiments were conducted at low ionic strength to see whether an increased interaction between FA–heparin and the 25 kDa Hep-1 fragment could be detected by the anisotropy

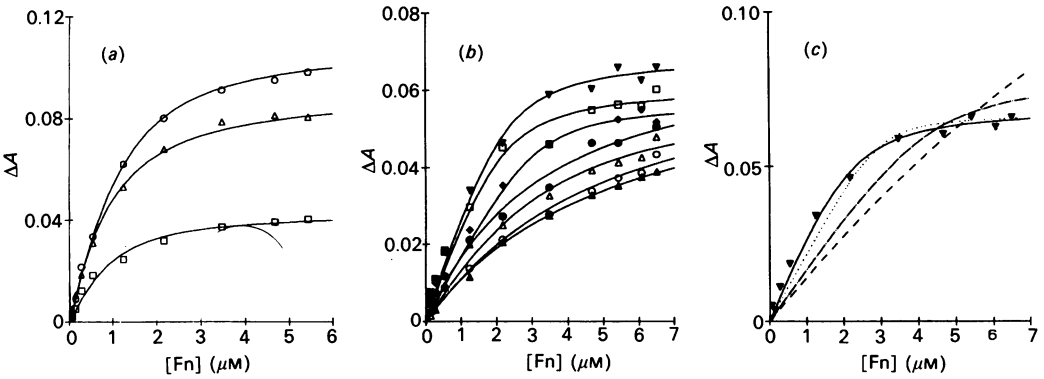

Fig. 2. Fn titration of fluoresceinamine-labelled heparin fractions

(*a*) FA–heparin fractions from Sephadex G-100 pools 1 (○), 4 (△) and 7 (□) were diluted with Tris-buffered saline, pH 7.4, to final concentrations of 4.3 μM, 2.3 μM and 3.2 μM respectively. The anisotropy was measured at 25 °C as a function of the concentration of added Fn. The continuous curves through the data points are theoretical best fits of the data to eqn. (1) with $n = 4$. The dissociation constants obtained in this manner are given in Table 1. (*b*) Titration of FA–oligosaccharides: 6-mer (▲); 8-mer (○); 10-mer (●); 12-mer (△); 14-mer (◆); 16-mer (□); 18–20-mer (▼). The labelled oligosaccharides obtained from chromatography on a polyacrylamide P-10 column (see the Materials and methods section) were diluted with Tris-buffered saline, pH 7.4, to final concentrations of 3–10 μM. The anisotropy was measured at 25 °C as a function of the concentration of added Fn. The curves through the data points are theoretical best fits of the data to the binding given by eqn. (1) with $n = 4$. The dissociation constants obtained in this manner are given in Table 1. (*c*) Titration of FA–18–20-mer oligosaccharide. Theoretical best fit of the data to eqn. 1 as a function of stoichiometry: $n = 1$ (----); $n = 2$ (·—·); $n = 3$ (·····); $n = 4$ (——). The labelled oligosaccharide obtained from chromatography on a polyacrylamide P-10 column (see the Materials and methods section) was diluted with Tris-buffered saline, pH 7.4, to a final concentration of 8.9 μM. The anisotropy was measured at 25 °C as a function of the concentration of added Fn.

Table 2. Summary of dissociation constants for binding of Fn and fragments of Fn to FA–heparin

Titrations were performed with 2 μM-FA–heparin pool 3; measurements were made in Tris-buffered saline, except at pH 6.0 and 8.0, where 0.02 M-Mes was included. K_d values were obtained by fitting titration data to eqn. (1) with $n = 4$ for Fn and $n = 2$ for the fragments of fibronectin.

Titrant	pH	Temperature (°C)	Concn. of NaCl (M)	K_d (μM)
Fn	6.0	25	0.15	1.2
	7.4	25	0.15	4.2
	8.0	25	0.15	49
	7.4	25	0.01	0.43*
			0.05	0.87
			0.10	4.0
			0.20	18
			0.30	30
Hep-2A fragment (30 kDa)	7.4	25	0.15	12
Hep-2B fragment (40 kDa)	7.4	25	0.15	15
Hep-1 fragment (25 kDa)	7.4	25	0.15	~ 300

* This value is considered an upper limit; the titration curve showed positive co-operativity, possibly due to an effect of low ionic strength on the structure of heparin.

method. In 0.02 M-Tris/HCl buffer with no added salt, Hep-1 fragment at concentrations above 1 μM caused a sharp increase in the anisotropy of FA–heparin. However, Hep-1 fragment was found to have limited solubility under these conditions, even in the absence of heparin, producing turbidity, which may have affected the measurements. Both the visual turbidity and the increase in anisotropy could be progressively reversed by addition of salt, the reversal being essentially complete at physiological ionic strength. Reversal could also be effected by Ca^{2+}, Mg^{2+} and Mn^{2+}, but only at ionic strengths close to those required for NaCl.

The contrast between Hep-1 and Hep-2 domains with respect to fluid-phase binding of heparin could also be demonstrated by exclusion chromatography. The continuous-line profiles in Fig. 4 represent the behaviour of Hep-1, Hep-2A and Hep-2B fragments on the Superose-12 column in Tris-buffered saline. The three fragments were eluted as sharp peaks centred at 13.8, 13.5 and 12.6 min respectively, consistent with their increasing size, with no evidence of aggregation or significant impurities. Mixing the fragments with excess heparin before application to the column produced the dashed-line profiles in Fig. 4. The elution of both Hep-2 fragments was shifted towards the void volume, whereas that of Hep-1 fragment was not significantly affected. Thus the Hep-1 fragment does not form a complex of sufficient stability to be detected by this method.

In a further attempt to detect a fluid-phase interaction with Hep-1 fragment, the exclusion column and eluting buffer were equilibrated with heparin at a concentration of 5 mg/ml (approx. 400 μM if the molecular mass is 13 kDa) before injection of a sample of Hep-1 fragment containing the same concentration of heparin. The dotted-line profile in Fig. 4(*c*) shows that under these conditions the elution of Hep-1 fragment shifted towards the void volume by an amount similar to that evoked with the Hep-2 fragments by merely adding much less heparin to the applied sample. Thus, although a fluid-phase complex can be demonstrated with Hep-1 fragment, the apparent affinity of this domain for heparin is much lower than that of the Hep-2 domains.

The contrast in apparent affinity of the Hep-1 and Hep-2 domains for heparin could also be demonstrated with immobilized fragments. When FA–Heparin was applied in Tris-buffered saline to a 1.5 ml column containing a mixture of the 30 kDa Hep-2A and 40 kDa Hep-2B fragments immobilized on Sepharose, more than 95% of the material bound. This is in sharp contrast with the situation with Hep-1 fragment–Sepharose, in which case about 95% of the fluorescent material passed through unretarded.

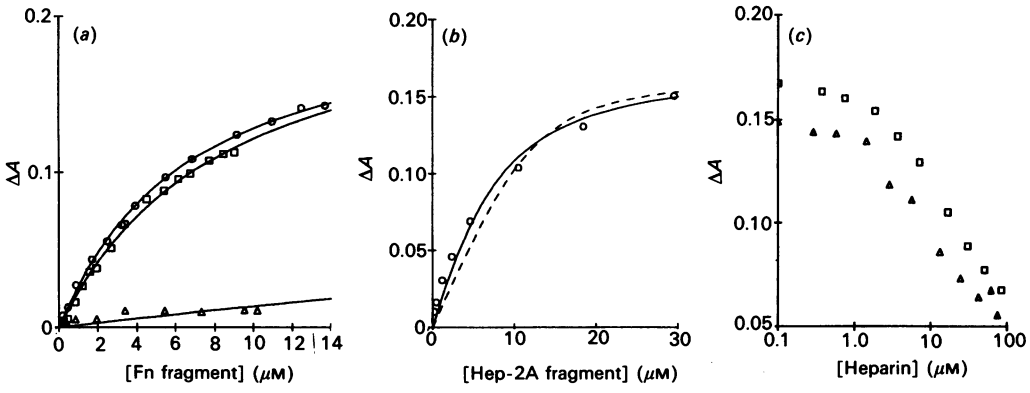

Fig. 3. Titration of FA–heparin with Fn fragments

(*a*) A sample of pool 3 (see Table 1) was diluted with Tris-buffered saline, pH 7.4, to a final concentration of 2.3 μM and the anisotropy was measured at 25 °C as a function of the concentration of added fragment. The continuous curves through the data points for 30 kDa Hep-2A fragment (○), 40 kDa Hep-2B fragment (□) and 25 kDa Hep-1 fragment (△) are theoretical best fits of the data to eqn. (1) with $n = 2$. Dissociation constants obtained in this way are summarized in Table 2. (*b*) Titration of 14-mer oligosaccharide with 30 kDa Hep-2A fragment. Theoretical best fit of the data to eqn. (1) as a function of stoichiometry: $n = 1$ (----); $n = 2$ (——). The labelled oligosaccharide was diluted with Tris-buffered saline, pH 7.4, to a final concentration of 12 μM. The anisotropy was measured at 25 °C as a function of the concentration of added Fn fragment. (*c*) Inhibition by unlabelled heparin of the binding of Hep-2 fragments of Fn to FA–heparin. Samples similar to those in (*a*), containing 7.4 μM 30 kDa Hep-2A fragment (△) or 5.9 μM 40 kDa Hep-2B fragment (□), were titrated with unlabelled heparin. The concentration of heparin on the ordinate is based on an average molecular mass of 13 kDa.

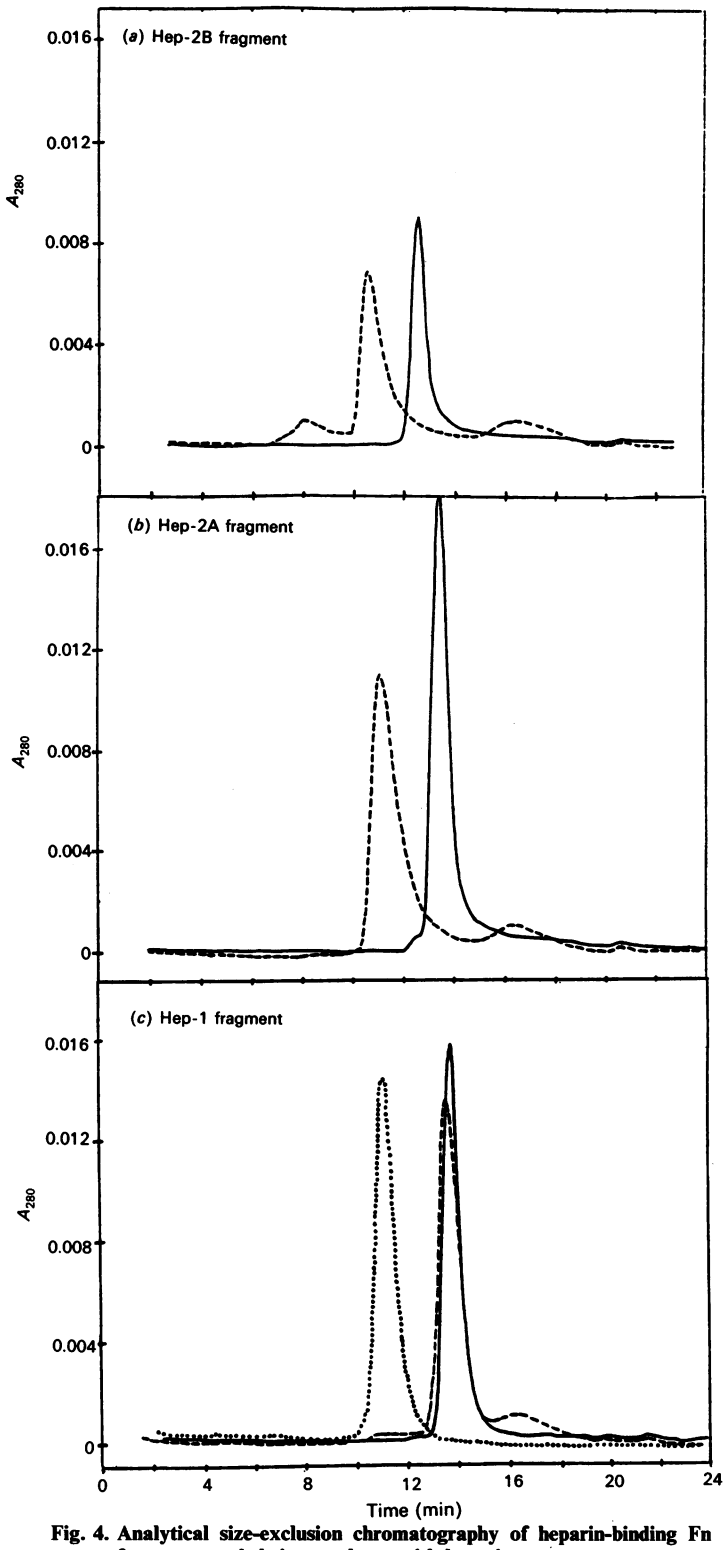

Fig. 4. Analytical size-exclusion chromatography of heparin-binding Fn fragments and their complexes with heparin

Samples (25 μl) of the fragments (0.5 mg/ml) were chromatographed on a Superose-12 column at 1 ml/min in Tris-buffered saline, pH 7.4, at room temperature alone (——) or premixed with an equal volume of heparin (10 mg/ml) (------). The dotted-line profile (·····) shows the elution of Hep-1 fragment when the entire column and eluting buffer contained 5 mg of heparin/ml.

DISCUSSION

Our measurements indicate that bulk heparin is relatively homogeneous with respect to its affinity for Fn. If multiple sites exist on the same heparin molecule, they are relatively independent and of similar affinity. The dissociation constants obtained in this study are in good agreement with that given by Evington *et al.* (1985) but substantially higher than those reported by Benecky *et al.* (1988) and Bentley *et al.* (1985) under similar conditions using the same technique. The difference might be attributable to the fact that, unlike the latter two groups, no particular attempt was made in the present study or in that by Evington *et al.* (1985) to select for a high-affinity population of FA–heparin. Our objective was to assess the binding properties of the entire population. In so doing, we found little evidence for significant heterogeneity in the binding to Fn at physiological ionic strength. However, at sub-physiological ionic strength, Evington *et al.* (1985) observed a 2-fold increase in affinity with increasing size of heparin from 6 to 10 kDa. Under these conditions they also observed a stoichiometry of four heparin chains to one fibronectin dimer.

Ogama *et al.* (1985) resolved several fractions of FA–heparin by stepwise elution from Fn–Sepharose with increasing concentrations of NaCl. Although no binding constants were determined, they noted a trend towards increasing size with increasing concentration of NaCl required for elution. We arrived at a different conclusion by fractionating first according to size and then systematically analysing the affinity of the different sized fractions for Fn. Under buffer conditions identical with those used by Ogama *et al.* (1985) the concentrations of NaCl required for gradient elution of the Sephadex G-100 pools from immobilized Fn lay within a narrow range, with no trend according to size. In a quantitative titration assay, K_d values for the different Sephadex G-100 pools lay within experimental variation of each other. Extension of this analysis to smaller fractions of heparin revealed a 5–10-fold decrease in affinity for oligosaccharides containing fewer than 14 saccharide groups.

The sensitivity of the K_d to ionic strength is consistent with an interaction dominated by electrostatic forces. The lack of temperature-dependence is also characteristic of such an interaction (Ross & Subramanian, 1981). The pronounced dependence of the binding on pH in the neutral range is consistent with the qualitative measurements of heparan sulphate binding to immobilized Fn published by Stamatoglou & Keller (1982). This result is difficult to reconcile with the absence of histidine residues in the Hep-2 domain. Perhaps the effect arises indirectly from secondary interaction of the Hep-2 domains with other domains of the Fn molecule that contain histidine residues. Fn is known to undergo rather large changes in hydrodynamic properties as a function of ionic strength and pH. Some workers have interpreted these changes in terms of disruption of intramolecular interactions between oppositely charged domains (Williams *et al.*, 1982; Markovic *et al.*, 1983), and others have suggested that heparin itself may disrupt those interactions (Hörmann & Richter, 1986).

The fluid-phase interaction of Fn with heparin is dominated by the Hep-2 domains. These domains, when isolated from the parent protein, elevated the anisotropy of FA–heparin almost as effectively as intact Fn, with apparent K_d values only slightly higher. The 30 kDa Hep-2A fragment was indistinguishable from the 40 kDa Hep-2B fragment in this respect; the presence of an additional type III homology unit in the larger fragment had no effect on the binding. The binding could be completely reversed by unlabelled heparin. This shows the fluorescent label still functions as a benign reporter of the interaction.

The weak anisotropy response obtained with 25 kDa Hep-1 fragment is consistent with the failure of heparin to induce a shift in the elution of the fragment in exclusion chromatography except at extremely high concentration, presumably because of the low affinity. An alternative interpretation that cannot be

excluded is that this fragment recognizes only a minor population of heparin molecules, as suggested by the low percentage of FA–heparin that bound to immobilized Hep-1 fragment under conditions where binding to immobilized Hep-2 fragment or intact Fn was quantitative. On the other hand, the Hep-1 fragment binds quantitatively to immobilized heparin and is eluted at about the same salt concentration as the Hep-2 fragment (see the Materials and methods section). This illustrates again the hazards of using heparin–Sepharose to assess relative affinity (Lennick *et al.*, 1986).

The dissociation constants in Tables 1 and 2 are based on a stoichiometry of 4 for fibronectin and 2 for the Hep-2 domain. These values are based on the results of Figs. 2(*c*) and 3(*b*) showing that titration data obtained with fluorescent oligomers could not be fitted with lower values of n. The oligomers are more reliable for this purpose because they reduce uncertainties arising from potential multiple binding sites on the larger heparins. The evidence that the Hep-2 domain has two binding sites for heparin is consistent with the value of $n = 4$ for Fn, given the low affinity of the Hep-1 domain.

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