Biochemical and Immunological Characterization of Three Binding Sites on Human Plasma Fibronectin with Different Affinities for Heparin†

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ABSTRACT: Three distinct heparin-binding regions have been isolated by heparin affinity chromatography from human plasma fibronectin (Fn) following limited proteolytic digestion with subtilisin. One fragment of molecular weight 29 000 exhibited low affinity for heparin, since it eluted from heparin–Sepharose with 0.1 M NaCl. The second region composed of three related fragments ranging in molecular weight from 29 000 to 36 000 demonstrated a high affinity for heparin, since they eluted with 0.5 M NaCl. A third bifunctional fragment of 22 000 daltons, which had been isolated by gelatin affinity chromatography, subsequently bound to heparin–Sepharose and eluted with 0.2 M NaCl, thereby demonstrating intermediate binding affinity for heparin. The heparin-binding fragments of low (SH29k), intermediate (SGH22k), and high affinity (SH29-36k) were further purified by high-pressure liquid chromatography. The sequence of the first 56 N-terminal amino acid residues of the SH29k fragment and the partial sequence of the SH29-36k fragments after CNBr cleavage were obtained. Since one sequence was obtained for the SH29-36k fragments, their molecular weight difference appears to be due to heterogeneity at the C terminal. Antiserum to the high-affinity SH29-36k fragments reacted faintly with intact Fn but did not react with the low-affinity SH29k fragment, while anti-fibronectin antiserum reacted with the SH29k fragment and intact Fn but not with the SH29-36k fragments. Therefore, it was concluded that the SH29k fragments and SH29-36k fragments represent two different heparin-binding domains and that the high-affinity site (SH29-36k fragments) is a less exposed antigenic site on the Fn molecule. We propose that the SGH22k fragment possessing intermediate affinity for heparin commences a maximum of 17 kdalton from the N terminal of the Fn molecule and extends into the N terminal of the gelatin- (collagen) binding domain. The SH29k fragment of low-affinity binding for heparin overlaps the C terminal of the collagen-binding domain at a region containing carbohydrate and shared antigenic determinants. The SH26-36k fragments of high affinity for heparin commence approximately 177 kdalton from the N terminal of the Fn molecule.

Fibronectin (Fn)[1] is a normal high molecular weight (M_r 440 000) glycoprotein constituent of blood and other body fluids. Fn also exists in an insoluble form on the surface of a variety of cells as a component of the extracellular matrix and in basement membranes and loose connective tissue. Fn is required for the adhesion of a variety of cells to collagen and to fibrin. The Fn molecule has specific binding sites for collagen, fibrin, heparin, hyaluronic acid, proteoglycans, and actin (Yamada & Olden, 1978; Pearlstein et al., 1980; Mosher, 1980; Mosesson & Amrani, 1980; Ruoslahti et al., 1981a; Kleinman et al., 1981).

Transformed cells that have decreased Fn on their surfaces acquire normal morphology following the addition of exogenous Fn derived from chick embryo fibroblasts (Yamada et al., 1976), possibly as a result of increased adherence to the substratum (Yamada et al., 1978). The ability of plasma Fn to mediate adhesion is also suggested by its activity as an opsonin during macrophage phagocytosis (Blumenstock et al., 1978). Fn can also be enzymatically cross-linked by plasma transglutaminase factor XIII to itself (Mosher, 1975), collagen (Mosher et al., 1980), or fibrin (Mosher, 1975). Both the enzymatic and noncovalent binding of Fn to these proteins, together with its chemotactic activity for fibroblasts (Czop et al., 1981) and monocytes (Bevilacqua et al., 1981), suggest a role for Fn in wound healing. Other activities ascribed to plasma Fn are binding to DNA (Hayashi & Yamada, 1982), Staph A (Mosher & Proctor, 1980), acetyl cholinesterase (Emmerling et al., 1981), and C1q (Pearlstein et al., 1982).

The myriad of binding activities attributed to Fn are localized within specific globular domains on the Fn molecule. Interdomain-extended regions susceptible to proteolytic cleavage have facilitated isolation of these biologically active regions on their respective ligands. Thus, the domains involved in binding to gelatin (collagen) (Gold et al., 1979), fibrin (Sekiguchi & Hakomori, 1980a), heparin (Hayashi & Yamada, 1982), actin (Keski-oja & Yamada, 1981), and DNA (Hayashi & Yamada, 1982) and the site involved in cell attachment (Piersbacher et al., 1981) have been isolated, partially characterized, and localized within the Fn molecule.

The present paper is concerned with the domains of Fn involved in heparin interaction. The formation of the Fn–fibrinogen cryoprecipitate requires heparin (Stathakis & Mosesson, 1977). Heparin also stimulates plasma Fn to form fibrillar aggregates, thereby enhancing its affinity for collagen (Ruoslahti & Engvall, 1980), especially to native collagen type III (Jilek & Hormann, 1979). Heparin and plasma Fn are necessary for the phagocytosis of gelatinized particles by Kupfer cells and macrophages (Blumenstock et al., 1978) and for the binding of type III collagen to macrophages (Hormann & Jelinic, 1981). Our studies demonstrate that Fn has three separate sites for heparin binding and that each site exhibits either a low, intermediate, or high affinity of binding to this ligand. Fragments obtained following subtilisin digestion of intact Fn were purified by affinity chromatography and HPLC. The N-terminal amino acid sequence of the low- and

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[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; TFA, trifluoroacetic acid; Fn, fibronectin; PTH, phenylthiohydantoin; IAA, iodoacetic acid; PBS, phosphate-buffered saline; DTT, dithiothreitol; HPLC, high-pressure liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate.

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high-affinity heparin-binding sites is presented, and a more precise localization of all three sites within the Fn molecule, than previously reported, is described.

Materials and Methods

Isolation of Human Plasma Fibronectin. Fn was prepared from human fresh frozen or outdated plasma by affinity chromatography on gelatin (swine skin, type I, Sigma) coupled to Sepharose 4B (3.0 mg/mL, Pharmacia) with cyanogen bromide (Pierce) (Cuatrecasas et al., 1968), as previously described (Ruoslahti & Engvall, 1978), except that the column was equilibrated with 0.075 M Tris, which increased Fn yields.

Subtilisin Digestion of Fn. Fn in 20-mg aliquots was incubated with subtilisin Carlsberg type VIII (Sigma) for 85 min at an enzyme substrate ratio of 1:100 in 1.0 mL of 0.1 M Tris–0.02 M $CaCl_2$, pH 8.2, at 37 °C as previously described (Gold et al., 1979). The reaction was terminated by the addition of PMSF (Sigma) and trasylol (Calbiochem) to a final concentration of 10^{-4} M and 25 units/mL, respectively. Five aliquots of digested protein were pooled on ice and applied directly to a gelatin–Sepharose 4B affinity column. The column was washed with 0.1 M Tris, pH 7.5, and the unbound material collected.

Heparin Affinity Chromatography. Heparin–Sepharose was purchased from Pharmacia. The resin was equilibrated in 0.05 M Tris, pH 7.5, and recycled after use by washing with 5 volumes of 0.05 M Tris containing 0.5 M NaCl and finally with 0.05 M Tris. Unbound fragments derived from chromatography on gelatin–Sepharose were diluted with distilled H_2O (1:1) and applied to the heparin–Sepharose. The column was eluted stepwise with buffers of 0.05 M Tris, pH 7.5, containing 0.1, 0.2, and 0.5 M NaCl. Each eluent was dialyzed separately against distilled H_2O and lyophilized.

Purification of the Heparin-Binding Fragments by Reverse-Phase High-Pressure Liquid Chromatography (HPLC). Approximately 0.5–1.0 mg of the lyophilized fragment to be purified was dissolved in 500 μL of 0.05% TFA, pH 2.4 (solution A), and clarified by centrifugation. A Waters HPLC (Model 660) equipped with a C_{18} preparatory μBondapak column (57 mm × 30 cm) was used for all chromatographic procedures. Elution of the fragments was achieved by a linear gradient from solution A to solution B (0.05% TFA–acetonitrile, 1:3). All samples were lyophilized directly and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. After it was determined at which points the fragments were eluted, a stepwise elution was routinely performed.

Chemical Cleavage of the Heparin-Binding Fragments by Cyanogen Bromide (CNBr). HPLC-purified fragments were dissolved in 70% formic acid at 4.0 mg/mL and mixed with an 8:1 excess of CNBr (Pierce). After incubation at room temperature for 48 h, the solution was diluted with 5 volumes of distilled water and lyophilized. Prior to amino acid sequence analysis, the small peptide(s) was (were) removed by dialysis in spectropore M_r 8000 cutoff dialysis membranes.

Amino Acid Analysis. Amino acid compositions of the fragments were determined in a Durrum D-500 amino acid analyzer following acid hydrolysis of lyophilized material with 0.2 mL of 6.0 M HCl at 110 °C for 18 h in vacuo, in the presence of phenol (40 μL of a 10% aqueous solution). Duplicate samples were oxidized with performic acid for 2.5 h at 4 °C prior to acid hydrolysis for quantitation of cysteic acid (Hirs, 1956).

Amino Acid Sequence Analysis. Amino-terminal analysis was performed by the manual Edman degradation technique (Gray, 1972). N-Terminal amino acid sequence was obtained by automated amino acid sequence analysis in 0.1 M Quadrol buffer, using an 890C sequence analyzer equipped with a P-6 sequemat autoconverter. One-tenth of the total volume of the PTH derivatives was identified by a Waters HPLC (Model ALC/GPC-204) equipped with a 3.9 mm × 30 cm C_{18} μBondapak column. The remaining nine-tenths of the sample was back hydrolyzed in 0.2 mL of 6 N HCl containing 0.005 mL of 1% stannous chloride in vacuo and analyzed on a Durrum D-500 amino acid analyzer (Michaelsen et al., 1977).

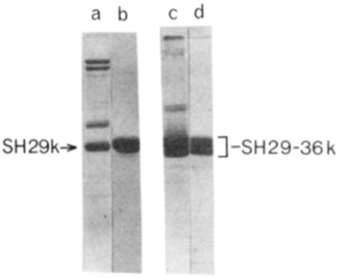

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (12.5% acrylamide) of subtilisin-digested fibronectin chromatographed on heparin–Sepharose: (lane a) fragments eluting with 0.05 M Tris containing 0.1 M NaCl (SH29k); (lane b) HPLC-purified SH29k; (lane c) fragments eluting with 0.05 M Tris containing 0.5 M NaCl (SH29-36k); (lane d) HPLC-purified SH29-36k.

Reduction and Alkylation of Fn. (*a*) *Partial Reduction.* Fn (1.0 mg) was partially reduced with DTT (final concentration of 20 mM) in 0.275 M Tris, pH 8.0, for 1 h at room temperature. The protein was then alkylated with IAA (Sigma) (final concentration 50 mM) under the same conditions. Free IAA was removed by dialysis against PBS.

(*b*) *Complete Reduction.* Fn was solubilized in 4.0 M urea–0.075 M Tris (pH 7.5) and reduced and alkylated as described above.

Immunodiffusion. Antiserum to the SH29-36k fragments was raised in rabbits, as previously described for Fn (Pearlstein & Gold, 1978). Double diffusion was performed according to Ouchterlony (1958), using specific antiserum to Fn and to the fragment.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed in slab gels as described by Laemmli (1970). Molecular weights of fragments were estimated by comparison with the following: phosphorylase *b*, M_r 68 000; ovalbumin, M_r 43 000; soybean trypsin inhibitor, M_r 21 000; and α-lactalbumin, M_r 14 000.

Results

Identification of Three Proteolytic Fragments from Fn with Different Heparin-Binding Affinities. (*1*) *Subtilisin Digestion of Human Plasma Fibronectin and Heparin Affinity Chromatography.* Digested Fn was chromatographed on gelatin–Sepharose; unbound fragments were diluted 2-fold with distilled water and applied to an affinity column containing 20.0 mL of heparin–Sepharose. The column was washed with 0.05 M Tris, pH 7.4, until the OD_{280} reached zero, and fragments were initially eluted with a gradient of NaCl to establish the minimum ionic strength required to elute each fraction. Subsequently, fragments were eluted stepwise with 0.05 M Tris (pH 7.4) containing 0.1, 0.2, and 0.5 M NaCl, respectively. Figure 1 shows $NaDodSO_4$–polyacrylamide gel electrophoresis of the fragments eluting with 0.1 M NaCl (lane a) and 0.5 M NaCl (lane c). Material eluting with 0.2 M NaCl contained small amounts of inconsistent fragments (not shown), and therefore, this step was used for washing.

(*2*) *Purification of Heparin-Binding Fragments by HPLC.* The heparin-binding fragments were further purified by re-

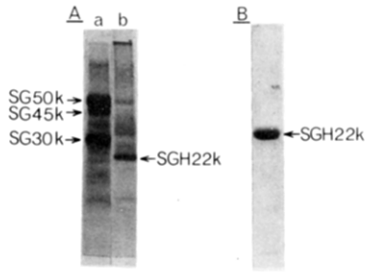

FIGURE 2: (A) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (12.5% acrylamide) of gelatin-binding fragments chromatographed on a heparin–Sepharose affinity column: (lane a) unbound fragments present in the effluent wash (the predominant SG50k, SG45k, and SG30k fragments are noted); (lane b) bound fragments eluting with 0.05 M Tris–0.2 M NaCl (the predominant band at M_r 22 000 is noted with an arrow). (B) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (14% acrylamide) of the gelatin–heparin-binding fragment SGH22k purified by HPLC.

verse-phase HPLC on a hydrophobic resin. A major fragment from the 0.1 M eluent (heparin affinity column) of M_r 29 000 (SH29k) was eluted from the HPLC column at a mixture of 51% solution A and 49% solution B (Figure 1, lane b). From the 0.5 M eluent, a triplet ranging in molecular weight from M_r 29 000 to M_r 36 000 (SH29-36k) was eluted from the HPLC at a mixture of 53% solution A and 47% solution B (Figure 1, lane d). The SGH29-36k could not be resolved into single polypeptides by ion-exchange chromatography or chromatography on the hydrophobic resin.

(3) Isolation of a Fragment That Binds to both Heparin and Gelatin. Gelatin-binding fragments eluted from gelatin–Sepharose with 4.0 M urea in 0.1 M Tris, pH 7.5, were dialyzed and lyophilized. The fragments (5.0 mg) were then solubilized in 2.0 mL of 0.1 M acetic acid, dialyzed overnight against 0.05 M Tris, pH 7.4, and applied to a column containing 10.0 mL of heparin–Sepharose. Both effluent and eluent were dialyzed against water and lyophilized for Na-DodSO_4–polyacrylamide gel electrophoretic analysis.

It was found that less than 10% of the gelatin-binding material applied to the column bound to the heparin–Sepharose. Figure 2A illustrates that the major gelatin-binding fragments of M_r 50 000 (SG50k), 45 000 (SG45k), and 30 000 (SG30k) (Gold et al., 1979) did not bind to the heparin column and were present in the effluent wash (Figure 1A, lane a). Protein was not eluted from the heparin column with 0.1 M NaCl; however, 0.2 M NaCl eluted a peptide of M_r 22 000 (Figure 2A, lane b) designated SGH22k (for both binding activities). The SGH22k was further purified by HPLC and eluted from the column at a mixture of 62% solution A and 38% solution B (Figure 2). Manual amino acid sequence analysis indicated Gly-Glx as the first two N-terminal amino acids.

Amino Acid Composition of Heparin-Binding Fragments. Comparative amino acid analyses of the purified heparin-binding fragments and intact Fn are given in Table I. Most noteworthy is the fact that both the SH29k and SH29-36k fragments are devoid of half-cystine, while there is a greater enrichment of this amino acid in the SGH22k fragment compared to that in intact Fn.

N-Terminal Amino Acid Sequence of SH29-36k and SH29k Domains. Automated sequence analysis of the SH29-36k high-affinity fragments permitted sequencing up to residue 34 (Figure 3). CNBr cleavage of the two methionines present in the SH29-36k fragments (Table I) produced three fragments with a molecular weight approximately 6000 less than that of the uncleaved polypeptides (data not shown). Automated sequence analysis of the cleaved and

Table I: Comparison of Amino Acid Composition[a] of Heparin-Binding Fragments with Intact Plasma Fibronectin

residue	Fn[b]	SGH22k	SH29k	SH29-36k
Asp	10.0	11.2	6.9	7.6
Thr	10.0	9.6	10.4	14.5
Ser	8.4	8.4	6.4	5.9
Glu	11.6	13.6	10.4	9.1
Pro	8.0	8.9	9.6	10.0
Gly	8.8	14.8	7.6	5.8
Ala	4.5	6.3	5.6	7.1
Val	7.9	6.1	11.6	9.4
Met	1.1 (24)[c]	1.5 (2.7)	0.4 (1.0)	0.7 (1.8)
Ile	4.6	4.5	3.1	5.9
Leu	5.7	7.1	7.4	6.8
Tyr	4.8	4.5	4.2	4.0
Phe	2.9	2.5	2.7	1.2
His	2.2	2.6	1.2	0
Lys	3.8	5.6	4.0	5.3
Arg	6.2	7.6	6.9	6.4
$^1/_2$-Cys[d]	2.0 (44.2)	4.7 (8.2)	0	0

[a] Expressed as mol %. [b] Computed on the basis of the monomeric molecular weight of 220 000. [c] Numbers in parentheses are expressed as mol/mol. [d] Determined as cysteic acid.

FIGURE 3: N-Terminal amino acid sequence of the high-affinity SH29-36k heparin-binding fragments compared to a homologous region of bovine Fn sequenced by Petersen et al. (1983). Methionines are circled. Amino acid differences are boxed. Since only two methionines (Table I) were found in the SH29-30k fragments, the segments S6, S7, and S8 obtained by Petersen et al. (1983) are arranged in tandem (denoted by *). Residue enclosed in parentheses is tentative.

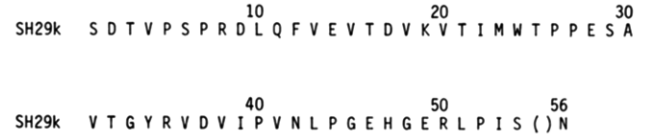

FIGURE 4: N-Terminal amino acid sequence of the low-affinity heparin-binding fragment (SH29k). ( ) indicates unidentified.

dialyzed fragments allowed additional sequencing up to residue 100 (68 residues in total) (Figure 3).

We have obtained homology between our sequence and that obtained by Petersen et al. (1983) for fragments of bovine Fn (Figure 3). The 17 residues between the two methionines were probably lost following dialysis after CNBr cleavage. The difference in molecular weight of the three fragments comprising the SH29-36k is apparently due to heterogeneity at the C terminal of the fragments.

The N-terminal amino acid sequence of the SH29k low-affinity heparin-binding fragment is shown in Figure 4. The sequence was determined by automated sequence analysis of intact SH29k and of protein purified following CNBr cleavage of the methionine at position 23 from the N terminal of the fragment. This sequence is identical with that of a 24-kdalton DNA-binding tryptic fragment from human plasma obtained by Pande & Shively (1982; personal communication).

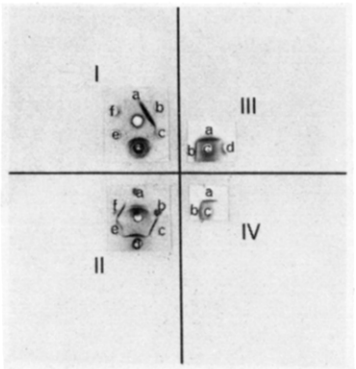

FIGURE 5: Immunodiffusion in 1% agarose gels of Fn and subtilisin fragments. (I) (Center well) Rabbit anti-SH29-36k; (a) SH29k; (b) SH29-36k; (c) native Fn; (d) human plasma; (e) partially reduced fibronectin; (f) completely reduced fibronectin. (II) (Center well) Rabbit anti-Fn; (a–f) as in (I). (III) (a) SH29-36k; (b) subtilisin-digested Fn; (c) rabbit anti-SH29-36k; (d) native Fn. (IV) (a) SH29k; (b) SH50k; (c) rabbit anti-Fn.

Immunodiffusion Studies. To more firmly establish that the SH29k and SH29-36k fragments are derived from different regions of the Fn molecule, immunologic studies were performed. Rabbits were immunized with purified SH29-36k. Figure 5I shows that anti-SH29-36k antiserum in the center well reacted with the SH29-36k fragments (b) and faintly with intact Fn (c) but not with the SH29k fragment (a). In Figure 5II, anti-Fn antiserum was used in the center well. A precipitin line can be seen between the SH29k fragment (a) and anti-Fn antiserum, but there was no reaction between the SH29-36k fragments (b) and anti-Fn. Because the reaction between the anti-SH29-36k antiserum and Fn was absent, it was thought that denaturing the molecule by reduction and alkylation might better expose this determinant. Figure 5I shows that if Fn was completely reduced and alkylated, it formed a weak precipitin band (f) with anti-SH29-36k antiserum, while no reaction could be seen between partially reduced and alkylated Fn (e) and the antiserum. Anti-Fn reacted with both partially and completely reduced Fn (Figure 5II, e and f). As expected, a reaction of partial identify was observed when anti-Fn antiserum was reacted with SH29k and reduced intact Fn in adjacent wells (Figure 5II, a and f). In Figure 5I, anti-SH29-36k antiserum reacted with plasma (d) containing Fn but weakly with purified Fn (c), although both contained equal concentrations of Fn. In Figure 5III, it is further shown that anti-SH29-36k antiserum reacted with subtilisin-digested Fn (b) but, again, only weakly with intact purified Fn (d).

When anti-Fn antiserum was reacted with purified SH29k fragment and a previously described (Gold et al., 1979) fragment of Fn of 50 000 daltons, which demonstrates gelatin-binding activity (SG50k), a reaction of identity was observed between the SH29k (Figure 5IV, a) and the SG50k fragments (Figure 5IV, b). Thus, it appears that although the SG50k polypeptide exclusively binds to gelatin (Gold et al., 1979) and the SH29k only binds to heparin, they both share an antigenic determinant that is recognized by the anti-Fn antisera.

Discussion

Three heparin-binding regions have been identified, biochemically characterized, and localized within the Fn molecule. Each of the isolated regions displayed different affinities of binding to heparin. One fragment of M_r 29 000 (SH29k) showed a low affinity of binding to heparin, another of M_r 22 000 (SGH22k) had an intermediate binding affinity, and a closely spaced triplet of M_r 29 000–36 000 (SH29-36k) exhibited a high affinity for heparin.

SH29k Fragment. The SH29k fragment eluted from heparin–Sepharose with 0.1 M NaCl and therefore displayed a low affinity of binding to heparin. This fragment also stained positively for PAS and thus contains carbohydrate. Sekiguchi et al. (1981) and Fukuda et al. (1982) have shown that the collagen-binding domain of hamster plasma Fn contains most and probably all of the carbohydrate present in the intact molecule. Although species differences have been noted for the carbohydrate composition of Fn (Ruoslahti et al., 1981a; Pearlstein et al., 1980), we have localized nearly all of the carbohydrate in human plasma Fn to a cyanogen bromide fragment of 18K from the C terminal of the SG50k gelatin-binding fragment (Figure 6; unpublished results). Because of the above and a reaction of partial identity (Figure 5IV) between SH29k and SG50k with antisera to Fn, it seems likely that the SH29k fragment is localized at the carboxyl end of SG50k.

The region of low-affinity heparin binding on human plasma Fn has not been described previously since most investigators have applied Fn digests to heparin affinity columns at salt concentrations above 0.1 M NaCl (Hayashi & Yamada, 1982, 1981; Smith & Furcht, 1982; Erishmann et al., 1982; Ruoslahti et al., 1981b) or at low salt concentrations but using different enzymes (Richter et al.,1981).

Pande & Shively (1982) isolated a tryptic 24-kdalton fragment from human plasma Fn by DNA affinity chromatography that had an identical N-terminal cleavage point and amino acid sequence as did the SH29k fragment present in these studies (Figure 4). Since heparin affinity chromatography preceded the DNA affinity column and the digested material was applied to heparin in a buffer of 0.04 M NaCl–25 mM Tris, pH 7.4, it is not readily apparent why the 24-kdalton fragment was not detected. Furthermore, it was shown by Hayashi & Yamada (1981) that all Fn fragments derived by thermolysin digestion that exhibits DNA-binding affinity also bind to heparin, while all heparin-binding fragments did not bind to DNA. This discrepancy may be explained by the difference in molecular weight of the DNA-binding fragment of 24K (Pande & Shively, 1982) and our heparin-binding fragment of 29K.

The isolation of the SH29k heparin-binding and 24K DNA-binding fragment (Pande & Shively, 1982) has now defined a biologically active domain that is part of the region of the Fn molecule between the end of the collagen-binding domain and the beginning of the "cell-spreading" domain (an area of 50–120 kdalton) (Ruoslahti et al., 1981b), which previously did not have an ascribed function.

SGH22k Fragment. The SGH22k fragment that bound to gelatin–Sepharose also bound to heparin–Sepharose and was eluted with 0.2 M NaCl (Figure 2, lane b), thus displaying an intermediate affinity for binding to heparin. Apparently, the SGH22k fragment is a minor cleavage product since the yields were very poor at approximately less than 0.5% of the starting material. Thus, following its purification, only enough material for amino acid analyses and the sequence of the first two N-terminal residues of Gly-Glx was obtained. The presence of a protease-sensitive site preceding the collagen-binding domain (Gold et al., 1979) could be responsible for the low yield of the SGH22k fragment.

SH29-36k Fragments. A cluster of three fragments of approximately 29K–36K demonstrated the highest affinity for binding the heparin–Sepharose since 0.5 M NaCl was required for their elution (Figure 1, lane c). Attempts to separate the three fragments by ion-exchange chromatography, hydro-

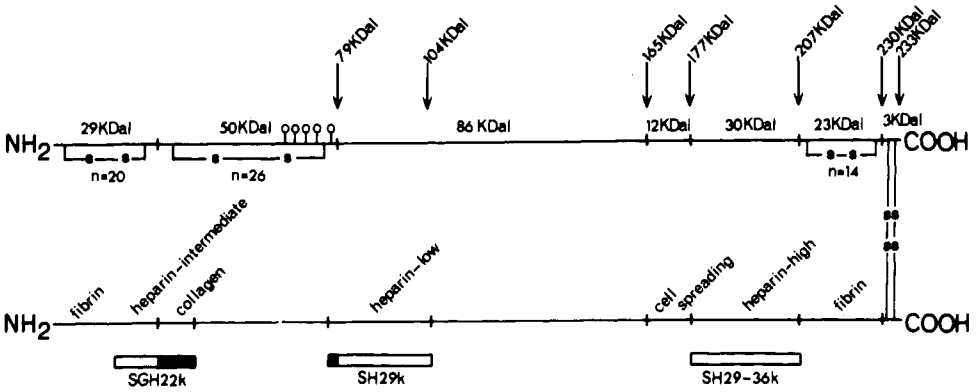

FIGURE 6: Topographical model localizing the heparin binding and other sites on Fn. Domains possessing intrachain disulfides are marked with S–S. The subunits of Fn are presumed to be identical and covalently bound by two interchain S–S bonds (Petersen et al., 1983). The upper chain indicates the map length (kdalton) of each active fragment, and the arrows above indicate distances from the N terminal of the Fn molecule. The biological activities corresponding to the indicated map units are presented on the lower chain. The individual biologically active fragments are shown below with overlapping areas shaded. The nomenclature devised for the fragment is as follows: The first letter designates the enzyme used for digestion, the second (or third) letter(s) designate(s) the ligand that the fragment has affffinity for, and the number designates the molecular weight(s) of the fragment(s) in kdaltons. The activities are presented ordered from N terminal to C terminal on the lower chain. The factor XIII cross-linking site is at the third amino acid from the N terminal (Q) (McDonagh et al., 1981). Fibrin-binding activity precedes heparin-binding activity in the N terminal (unpublished observation). (O) Carbohydrate present is in a cyanogen bromide fragment of the SH50k representing the C terminal of this domain. Erishmann et al. (1982) have suggested a space of 93 kdalton between the end of their gelatin-binding domain of 43 kdalton and the beginning of the site mediating cell spreading. Since SG50k is 7 kdalton longer than their 43K fragment, the cell attachment site is approximately 86 kdalton from the end of SG50k or 165 kdalton from the N terminal of the whole molecule (29 kdalton + 50 kdalton + 86 kdalton). The cell-spreading site is found in a fragment of 12 kdalton (Erishmann et al., 1982), and a chymotryptic 135-kdalton fragment contains both the cell-spreading site and the C-terminal heparin-binding site, while a chymotryptic fragment of 105 kdalton only possesses the cell-spreading site. Since these two fragments have the same N-terminal cleavage site (Erishmann et al., 1982), the C-terminal heparin-binding site (SH29-36k high-affinity site) is a maximum of 30 kdalton from the cell-spreading site. Thus, from the N terminal of Fn to the end of the high-affinity heparin site is 207 kdalton (165 kdalton + 12 kdalton + 30 kdalton). C terminal to the 207-kdalton area is a domain of 23 kdalton and then one of 3 kdalton derived from plasmin digestion of Fn (Petersen et al., 1983). The cell-spreading area has been confined to the C terminal of the 12 kdalton area (Piersbacher et al., 1983) and is the last 30 residues of the S9 fragment described by Petersen et al. (1983).

phobic chromatography, or isoelectric focusing methods proved unsuccessful. However, we have obtained a single sequence with the SH29-36k polypeptides, and it therefore appears that the three polypeptides contain one heparin binding site and are heterogeneous at their C-terminal ends. Several investigators have localized this site to be toward the C terminal of the Fn molecule (Smith & Furcht, 1982; Sekiguchi & Hakomori, 1980b; Richter et al., 1981; Ruoslahti et al., 1981b).

Various heparin-binding sites have been characterized by Hayashi & Yamada (1982) according to their insensitivity and/or sensitivity to the divalent cations calcium and magnesium. These authors showed that their N-terminal heparin-binding site, which is the same binding site within the SGH22k fragment, is calcium sensitive in the presence of 0.1 M NaCl and that a tryptic fragment of 95K was cation insensitive (the same as the SH29-36k fragment reported here) and eluted from heparin only with 0.5 M NaCl. A third site, which was positioned C terminal to the cation-insensitive site, was recovered in a tryptic fragment of 75K that was found to be both calcium and magnesium sensitive. Enzymatic digestion with subtilisin did not liberate this heparin-binding site, and if we combine our data with those of Hayashi & Yamada (1982), there would be a total of four separate heparin-binding domains within the human plasma Fn molecule including the low-affinity heparin-binding site (SH29k fragment) reported in these studies.

Comparison of the Heparin-Binding Fragments. Immunodiffusion studies illustrated that anti-SH29-36k antiserum reacted strongly with the SH29-36k fragments (Figure 5I), did not react with the SH29k fragment, and only reacted weakly with intact Fn with or without complete reduction and alkylation (Figure 5I). Antithetically, anti-Fn antisera showed a strong reaction with the SH29k fragment but did not react with SH29-36k fragments (Figure 5II). Certain inferences can be made from these results that are consistent with the biochemical data presented: (1) The SH29k fragment and SH29-36k fragments are not from the same heparin-binding region and do not share antigenic determinants. Therefore, they represent two different heparin-binding domains on the Fn molecule; this is also suggested by the sequence data. (2) Only one precipitin line was observed between the anti-SH29-36k antiserum and the SH29-36k fragments. Thus in agreement with the sequence data, these three fragments contain the same heparin-binding site. (3) The high-affinity heparin-binding site (SH29-36k fragments) is a less exposed site on the Fn molecule than the low-affinity site (SH29k fragment). This is proposed because (a) intact Fn used for immunization elicited antibodies to determinants on the SH29k fragment but not the SH29-36k fragments (Figure 5II), although the isolated SH29-36k fragments were immunogenic (Figure 5I), and (b) subtilisin-digested Fn reacted strongly with anti-SH29-36k antiserum (Figure 5III) but not intact Fn. Furthermore, since anti-SH29-36k antiserum formed a precipitin line with freshly collected plasma but reacted only weakly with purified Fn (containing an equivalent concentration of Fn, Figure 5I), Fn may circulate in the plasma in a particular tertiary conformation exposing this site, perhaps as a result of binding to another molecule. Alternatively, this heparin-binding site may exist in plasma as a natural proteolytic cleavage product.

Chemical cleavage of the SH29k low-affinity heparin-binding fragment and SH29-36k high-affinity heparin-binding fragment with CNBr yielded large fragments with molecular weights of 26 000 and 22 000–29 000, respectively. These fragments retained their respective heparin-binding activities. Moreover, CNBr cleaved the one methionine in the SH29k fragment at the N-terminal 23rd residue (Figure 4) and the second N-terminal methionine at position 66 of the SH29-36k fragment (Figure 3). Therefore, heparin-binding activity is not within the first 23 or first 65 residues of the SH29k and

SH29-36k fragments, respectively. From a combination of the human plasma Fn data from these studies and bovine plasma Fn data presented by Peterson et al. (1983), 40% of this heparin-binding domain has currently been sequenced. Apparently, there are only four amino acid differences between the human and bovine high-affinity heparin-binding region of Fn sequenced to date (Figure 3, boxed amino acids).

A Topographical Model of Heparin-Binding Sites. The SGH22k fragment of intermediate binding affinity for heparin is placed near the N terminal of the Fn molecule for the following reason: the actual binding site on the collagen domain has recently been localized to be within the first 5–10 kdalton of this domain (Smith & Furcht, 1982). Since the SG50k and SG30k fragments, which possess the same N terminal and commence at the same site on the Fn molecule, only bind to gelatin and not heparin (Figure 2, lane a), a fragment of 22 kdalton (SGH22k) could not be within the C terminal of the SG50k (C terminal, 20 kdalton) and possess both of these activities. For this reason, the SGH22k fragment must be from a region immediately N terminal to the gelatin-binding domain, and more precisely, if it encompasses the first N-terminal 5 kdalton of SG50k, then the SGH22k heparin–gelatin-binding fragment can only extend a maximum of 17 kdalton toward the N terminal or 13 kdalton from the N terminal of the Fn molecule. The first two N-terminal amino acid residues of SGH22k are Gly-Glx; therefore, placement of this fragment within the N terminal of the Fn molecule might be at positions 114–115, 144–145, or 167–168 (A. Garcia-Pardo et al., unpublished results). Moreover, the first N-terminal 80 kdalton of the Fn molecule is a region very rich in half-cystines (Balian et al., 1979; Furie & Rifkin, 1980; Petersen et al., 1983) and, therefore, is consistent with the placement of the SGH22k fragment, which contains eight half-cystines.

Sekiguchi & Hakomori (1980b) and Sekiguchi et al. (1981) were the first investigators to report that the N terminal of Fn contained a heparin-binding region. More recently, this particular site has been described by various investigators using an array of proteases (Hayashi & Yamada, 1982; Pande & Shively, 1982; Hayashi & Yamada, 1981; Richter et al., 1981; Erishmann et al., 1982). The SH29k low-affinity heparin-binding site is localized immediately C terminal to the collagen-binding domain (SG50k) with an overlap region containing the carbohydrate and shared antigenic determinant(s) (Figure 5IV).

The localization of the cell-spreading site on Fn (Erishmann et al., 1982) together with information contributed by Smith & Furcht (1982) using monoclonal antibodies to biologically active fragments of Fn have assisted in localizing the high-affinity (SH29-36k) heparin-binding domain to approximately 177 kdalton from the N terminal of the Fn molecule. A topographical model localizing the three heparin-binding domains of Fn is illustrated in Figure 6. The calculations presented in this model are consistent with Smith & Furcht (1982), who reported that a heparin-binding site is a minimum of 31 kdalton from the C terminal of the Fn molecule and that the entire length of the Fn subunit is betwen 220 and 250 kdalton (Yamada & Olden, 1978; Pearlstein et al., 1980; Mosher, 1980; Mosesson & Amrani, 1980; Ruoslahti et al., 1981a).

Of all the functions ascribed to Fn, only heparin-binding activity exists within three (or four) individual regions of the Fn molecule. That these regions demonstrate different affinities for binding to heparin and, as Hayashi & Yamada have shown (1982), are sensitive to cation modulation suggests that each different heparin-binding region of the Fn molecule may be involved in separate heparin-mediated functions and/or perhaps each binding site is directed toward a different portion of the heparin molecule or different heparin molecules entirely. Alternatively, further sequence data on the low-, intermediate-, and high-affinity heparin-binding fragments should determine whether there is a similar limited sequence area that dictates heparin-binding activity in all heparin-binding regions.

Registry No. Heparin, 9005-49-6.

References

Balian, G., Click, E., Crouch, E., Davidson, J. M., & Bornstein, P. (1979) *J. Biol. Chem. 254*, 1429–2432.

Bevilacqua, M. P., Amrani, D., Mosesson, M. W., & Bianco, C. (1981) *J. Exp. Med. 153*, 42–60.

Blumenstock, F. A., Weber, P., & Saba, T. M. (1978) *J. Reticuloendothel. Soc. 23*, 119–134.

Cuatrecasas, P., Wilcheck, M., & Afinsen, C. B. (1968) *Proc. Natl. Acad. Sci. U.S.A. 61*, 636–643.

Czop, J. K., Kadish, J. L., & Austen, F. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3649–3653.

Emmerling, M. R., Johnson, C. D., Mosher, D. F., Lipton, B. H., & Lilien, J. (1981) *Biochemistry 20*, 3242–3247.

Erishmann, R., Roth, D. E., Eppenberger, H. M., & Turner, D. C. (1982) *J. Biol. Chem. 257*, 7381–7387.

Fukuda, M., Levery, S. B., & Hakomori, S. (1982) *J. Biol. Chem. 257*, 6850–6860.

Furie, M. B., & Rifkin, D. B. (1980) *J. Biol. Chem. 255*, 3134–3140.

Gold, L. I., Garcia-Pardo, A., Frangione, B., Franklin, E. C., & Pearlstein, E. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4803–4807.

Gray, W. R. (1972) *Methods Enzymol. 25*, 121.

Hayashi, M., & Yamada, K. M. (1981) *J. Biol. Chem. 256*, 11292–11300.

Hayashi, M., & Yamada, K. M. (1982) *J. Biol. Chem. 257*, 5263–5267.

Hirs, C. H. W. (1956) *J. Biol. Chem. 219*, 611.

Hormann, H., & Jelinic, V. (1981) *Hoppe-Seyler's Z. Physiol. Chem. 362*, 87–94.

Jilek, F., & Hormann, H. (1979) *Hoppe-Seyler's Z. Physiol. Chem. 360*, 597–603.

Keski-oja, J., & Yamada, K. M. (1981) *Biochem. J. 193*, 615–620.

Kleinman, H., Klebe, R. J., & Martin, G. R. (1981) *J. Cell Biol. 88*, 473–485.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

McDonagh, R. P., McDonagh, J., Petersen, T. E., Thogersen, H. C., Skorstengaard, K., Sottrup-Jensen, L., & Magnusson, S. (1981) *FEBS Lett. 127*, 174–178.

Michaelsen, T. E., Frangione, B., & Franklin, E. C. (1977) *J. Biol. Chem. 252*, 883–889.

Mosesson, M. W., & Amrani, D. L. (1980) *Blood 56*, 145–158.

Mosher, D. F. (1975) *J. Biol. Chem. 250*, 6614–6621.

Mosher, D. F. (1980) *Prog. Hemostasis Thromb. 5*, 111–151.

Mosher, D. F., & Proctor, R. A. (1980) *Science (Washington, D.C.) 209*, 927–929.

Mosher, D. F., Schad, P. E., & Vann, J. M. (1980) *J. Biol. Chem. 255*, 1181–1188.

Ouchterlony, O. (1958) *Prog. Allergy 5*, 1–78.

Pande, H., & Shively, J. E. (1982) *Arch. Biochem. Biophys. 213*, 258–265.

Pearlstein, E., & Gold, L. I. (1978) *Ann. N.Y. Acad. Sci. 312*, 278–292.

Pearlstein, E., Gold, L. I., & Garcia-Pardo, A. (1980) *Mol. Cell. Biochem. 29*, 103–127.
Pearlstein, E., Sorvillo, J., & Gigli, I. (1982) *J. Immunol. 128*, 2036–2039.
Petersen, T. E., Thogersen, H. C., Skorstengaard, K., Vibe-Pedersen, K., Sahl, P., Sottrup-Jensen, L., & Magnusson, S. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 137–141.
Piersbacher, M., Hayman, E. G., & Ruoslahti, E. (1981) *Cell (Cambridge, Mass.) 26*, 259–267.
Piersbacher, M., Hayman, E. G., & Ruoslahti, E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1224–1227.
Richter, H., Seidl, M., & Hormann, H. (1981) *Hoppe-Seyler's Z. Physiol. Chem. 362*, 399–408.
Ruoslahti, E., & Engvall, E. (1978) *Ann. N.Y. Acad. Sci. 312*, 178–191.
Ruoslahti, E., & Engvall, E. (1980) *Biochim. Biophys. Acta 631*, 350–358.
Ruoslahti, E., Engvall, E., & Hayman, E. G. (1981a) *Collagen Relat. Res.: Clin. Exp. 1*, 95–128.
Ruoslahti, E., Hayman, E. G., Engvall, E., Cothran, W. C., & Butler, W. T. (1981b) *J. Biol. Chem. 256*, 7277–7281.
Sekiguchi, K., & Hakomori, S. (1980a) *Biochem. Biophys. Res. Commun. 97*, 709–715.
Sekiguchi, K., & Hakomori, S. (1980b) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2661–2665.
Sekiguchi, K., Fukuda, M., & Hakomori, S. (1981) *J. Biol. Chem. 256*, 6452–6462.
Smith, E. C., & Furcht, L. T. (1982) *J. Biol. Chem. 257*, 6518–6523.
Stathakis, N. E., & Mosesson, M. W. (1977) *J. Clin. Invest. 60*, 855–865.
Yamada, K. M., & Olden, K. (1978) *Nature (London) 275*, 179–184.
Yamada, K. M., Yamada, S. S., & Pastan, I. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1217–1221.
Yamada, K. M., Olden, K., & Pastan, I. (1978) *Ann. N.Y. Acad. Sci. 312*, 256–277.

Effect of Magnetic Susceptibility on Nuclear Magnetic Resonance Signals Arising from Red Cells: A Warning†

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ABSTRACT: We have demonstrated that magnetic susceptibility effects can strongly influence nuclear magnetic resonance spectra, particularly in cells, and lead to spurious line broadening and chemical shifts which will result in false conclusions about molecular motion and intracellular pH and equilibrium constants. Three specific instances of a general phenomenon resulting from the difference in magnetic susceptibility inside and outside deoxygenated red cells have been examined. We find that at 95 MHz the resonance of ^{19}F covalently bound to both hemoglobin and glutathione as the trifluoroacetonyl compound undergoes substantial line broadening in deoxygenated red cells. Line broadening is not observed in oxygenated solutions or oxygenated cells nor is it observed in deoxygenated hemoglobin solutions. The broad lines in the deoxy red cell can be narrowed by matching the magnetic susceptibility of the suspending medium to that of the cell interior by adding suitable amounts of a paramagnetic metal ion such as manganese or dysprosium to the suspending medium. Similarly, we have observed line broadening of the ^{31}P resonance of 2,3-diphosphoglyceric acid which occurs only in deoxygenated red cells and has a field dependence greater than the first power. This line broadening does not occur in oxygenated solutions or oxygenated cells nor does it occur in deoxygenated hemoglobin solutions. Again, the broadened lines in the deoxygenated cells can be narrowed by matching the magnetic susceptibility. Because the line-broadening effects reported here do not depend on the nucleus, the chemical nature of the molecule, or the molecular size, they cannot be attributed to specific chemical interactions or interactions with the cell membrane. Because they can be eliminated by matching the internal and external magnetic susceptibility, we attribute the observed broadening to magnetic susceptibility differences. Differences in magnetic susceptibility will also result in systematic displacement of the chemical shift of resonances arising inside the cell which may either add to or cancel effects due to biochemical interactions. We show that the appropriate corrections can be estimated but that due to the presence of magnetic field gradients use of internal references may be necessary.

Nuclear magnetic resonance (NMR)[1] is uniquely suited to the study of events occurring inside intact cells. It is frequently desirable to conduct such studies at high magnetic fields to overcome the problems of low signal to noise and overlapping resonances. However, if line-broadening effects occur which are more pronounced at high field, the expected enhancement in sensitivity and resolution will not be attained. Hemoglobin is present at high concentrations (5 mM) in the red cell and, particularly in its met and deoxy forms, has a magnetic susceptibility which differs significantly from that of water. Chemical shifts and line broadening due to magnetic susceptibility effects have been recognized in nonbiological systems since the earliest days of NMR. However, it has generally been assumed that such effects were negligible in biological

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[1] Abbreviations: 2,3-DPG, 2,3-diphosphoglyceric acid; Hb-TFA, hemoglobin with a trifluoroacetonyl group on both β-93 sulfhydryls; PBS, equal volumes of 0.15 M pH 7.2 phosphate buffer and isotonic saline; NMR, nuclear magnetic resonance; BTFP, 3-bromo-1,1,1-trifluoropropanone; PMB, *p*-(hydroxymercuri)benzoate; GSH, glutathione; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MCHC, mean corpuscular hemoglobin concentration; DTPA, *N*,*N*-bis[2-[bis(carboxymethyl)-amino]ethyl]glycine (diethylenetriaminepentaacetic acid); T_2, transverse relaxation time; T_1, longitudinal relaxation time.

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