Binding of fibronectin to gelatin and heparin: effect of surface denaturation and detergents

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The specific binding of fibronectin to collagen and heparan sulfate is important in cell adhesion and formation of connective tissue. We have used miniaturized affinity chromatography of ^{125}I-labeled fibronectin on gelatin- or heparin-conjugated Sepharose as a model system to further study features of these interactions after a variety of treatments of the fibronectin. We find that fibronectin's affinity is abolished following adsorption and desorption from tissue culture plastic, diminished by the strong ionic detergent sodium dodecyl sulfate (SDS), and unaffected by nonionic detergents or the zwitterionic detergent Zwittergent 3-12. This identifies the latter as a gentle extractant suitable for a variety of uses in studying cell-substratum adhesion.

Fibronectin *Collagen* *Heparan sulfate* *Affinity chromatography* *Detergent* *Adhesion*

1. INTRODUCTION

Cell surface fibronectin (Fn) and plasma fibronectin (pFn) are proteins which have aroused wide interest because of the great diversity of their functions and activities. They bind to glass and plastic surfaces, collagen, fibrin, certain glycosaminoglycans, and an unidentified cellular receptor, and are thereby involved in fibroblast adhesion and spreading in culture [1–5], macrophage adhesion [6], opsonization of bacteria [7], and wound healing (review [8,9]). Fn participates with collagen and heparan sulfate in the formation of the extracellular matrix of connective tissue, and is also notable for being diminished in many malignantly transformed cells [8,9].

Abbreviations: BSA, bovine serum albumin; Fn, fibronectin; ME, mercaptoethanol; PBS, phosphate-buffered saline (10 mM phosphate, pH 7.2); pFn, plasma fibronectin; SV-T2 cells, simian virus 40-transformed mouse fibroblasts; TMC, 50 mM Tris–HCl (pH 7.4), 1 mM $MgCl_2$, 1 mM $CaCl_2$; Tri, Triton X-100; Zw, Zwittergent 3-12

Here, we describe simple quantitative assays for the specific binding of Fn and pFn to gelatin (denatured collagen) and heparin, which use a miniaturized form of affinity chromatography. Since these measure simply and directly the fundamental binding properties of the fibronectins, they have many possible applications. They help, for example, to characterize the binding of Fn and pFn to an artificial surface, a process required before these proteins can promote cell adhesion. Fn and pFn, once adsorbed, are so firmly bound that only a few percent can be desorbed with buffer or even removed with detergent [10]. Our assays here demonstrate that the desorbed material is so denatured that it has lost most of its gelatin- and heparin-binding activity, further testifying as to how strongly the fibronectins were originally adsorbed to the surface.

The assays also permit simple rapid screening of the effect of chemical treatments, specifically here with detergents, on the fibronectins. Previous protein structure-function studies of this type [11,12] tested the ability of treated pFn to promote cell adhesion, a process requiring it to bind to both a

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collagen substratum and then to cell surface heparan sulfate proteoglycans; our assays complement these by helping identify precisely which of these two binding steps are affected.

2. MATERIALS AND METHODS

2.1. *^{125}I-labeled pFn*

pFn was isolated by gelatin–Sepharose affinity chromatography from heat-inactivated calf serum or outdated human plasma, radioiodinated enzymatically with immobilized glucose oxidase and lactoperoxidase (Enzymobeads, Bio-Rad), and repurified by gelatin–Sepharose affinity chromatography as in [10]. Approx. 0.3 atom ^{125}I was incorporated per molecule bovine pFn, the electrophoretic profile was similar to starting material, and ^{125}I-labeled pFn had cell spreading activity [10]. The ^{125}I-labeled pFn also retained its gelatin-binding activity (table 1, expt.A), and 80% bound to heparin columns (fig.1C), a value close to the 87% reported for unlabeled pFn [13].

2.2. *Surface-denatured pFn*

Plastic dishes (35 mm diameter, Lux) were coated with 5 μg bovine pFn containing 1.5×10^6 cpm ^{125}I-labeled bovine pFn, rinsed extensively, then incubated at 37°C for 2 h or 1 day with Eagle's minimal essential culture medium (supplemented with a 4-fold concentration of vitamins and amino acids) containing 1 mg/ml bovine serum albumin (BSA) with or without 4×10^6 SV-T2 cells added after the first hour [10]. (Under these conditions 1–4% of the bound pFn was released into the medium; analysis by SDS–polyacrylamide gel electrophoresis showed that the material included undegraded pFn, not just small protein fragments [10].) Aliquots (0.2 ml) of the medium (1500–10000 cpm), or of control ^{125}I-labeled pFn [150000 cpm (approx. 600 ng), containing 600 ng unlabeled carrier pFn], were subjected to affinity chromatography (below) on gelatin–Sepharose columns previously rinsed with 1 mg/ml BSA in Eagle's medium.

2.3. *Denaturant-treated pFn*

A 1 μl sample of ^{125}I-labeled human pFn (approx. 170 ng and 250000 cpm) was mixed with 0.2 ml denaturant [PBS; 0.2% SDS (Bio-Rad); 0.2% Zwittergent 3-12 (Calbiochem-Behring); 0.2% Triton X-100 (Sigma); 5 M NaCl; or 1% (0.14 M) mercaptoethanol], and allowed to stand at room temperature 10 min before affinity chromatography (below).

2.4. *Affinity resins*

Gelatin (Sigma) or heparin (Sigma) was coupled to Sepharose 4B (Pharmacia) activated with CNBr [14], using 20 mg gelatin or 5 mg heparin per ml resin, and underivatized sites were subsequently blocked with ethanolamine.

2.5. *Affinity chromatography*

2.5.1. Binding assays

To assay binding to gelatin, ^{125}I-labeled pFn was loaded on a 0.2 ml gelatin–Sepharose column contained in a 1 ml plastic syringe and previously rinsed with 0.8 ml phosphate-buffered saline (10 mM phosphate, 140 mM NaCl; pH 7.2) (PBS), 0.8 ml PBS (1 mg/ml in BSA), and then 0.8 ml PBS. It was rinsed into the column bed with a few drops of PBS, allowed to stand 10 min, and then eluted sequentially with two 0.8-ml vols each of PBS, 1 M urea (50 mM Tris, pH 7.5), and 4 M urea (50 mM Tris, pH 7.5). The radioactivity eluted in these 6 fractions, and that retained irreversibly on the column, was determined by gamma counting. Binding to heparin was determined similarly using a 0.2 ml heparin–Sepharose column previously rinsed with 0.8 ml TMC buffer [50 mM Tris–HCl (pH 7.4), 1 mM $MgCl_2$, 1 mM $CaCl_2$], 0.8 ml TMC (1 mg/ml in BSA), and 0.8 ml TMC; this was eluted sequentially with two 0.8-ml vols each of TMC, TMC (2 M NaCl), and TMC (5 M NaCl). The quantity eluted with 5 M NaCl was always less than 1% of the total.

2.5.2. Elution assays

Aliquots (1 μl) of ^{125}I-labeled human pFn were applied to 0.2-ml columns of gelatin–Sepharose or heparin–Sepharose (rinsed as described in section 2.5.1), allowed to stand 10 min at room temperature, then eluted sequentially with two 0.8-ml vols each of PBS (respectively TMC) and the indicated denaturant.

3. RESULTS

Quantitative affinity chromatography of untreated pFn (table 1, expt.A) confirmed the obser-

Table 1

Gelatin binding of pFn denatured by surface adsorption

Expt.	Conditions	Radioactivity eluted or bound (%)				Eluted by 4 M urea (% of total eluted)
		PBS	1 M urea	4 M urea	Column	
A	Control	18	5	39	38	64
		13	3	41	43	72
B	2 h, no cells	74	3	10	12	12
		74	2	11	13	13
C	2 h, with cells	72	6	12	10	14
		69	4	15	12	17
D	1 day, no cells	81	3	9	8	9
		81	3	8	8	9
E	1 day, with cells	75	4	11	10	13
		75	3	13	9	14

pFn denatured by adsorption to plastic for 2 h or 1 day, with or without the presence of SV-T2 cells, was applied to gelatin–Sepharose columns and eluted with PBS, 1 M urea, and 4 M urea (section 2.5.1). Radioactivity eluted or retained irreversibly on the columns is tabulated as percentage of total recovered. The bulk of the material labile to each extractant was eluted in the first of each pair of 0.8-ml fractions: more than 90% of it for PBS, 60% for 1 M urea, and 80% for 4 M urea. The radioactivity eluted with 4 M urea is also expressed as percentage of total eluted with PBS + 1 M urea + 4 M urea. The results of duplicate experiments are shown

vations in [15]: while a little pFn could be washed off a gelatin column by extensive elution (8 column vols) with PBS, and more with 1 M urea, only 4 M urea could remove the largest quantity (39–41% of input, or 64–72% of the total eluted). Once pFn had bound to tissue culture plastic (table 1, expts. B–E), however, the small portion that could be desorbed under any of the 4 conditions tested (2 or 24 h incubation with or without the presence of adherent cells) retained only about one-fifth of this ability to bind specifically to gelatin (or also to bind irreversibly to the column). Very similar results were obtained using chick cell surface Fn, the desorbed fibronectins also lost their affinity for heparin, and they similarly lost their affinity for both gelatin and heparin after adsorption-desorption from untreated hydrophobic ('bacteriological') polystyrene (not shown). The plastic surfaces, by attracting complementary hydrophobic regions of the fibronectins that are ordinarily buried deep within aqueously solvated molecules, have apparently unfolded and 'denatured' these intensely adhesive proteins in a way not unlike that resulting from detergent treatment [16–19].

To study the effects of detergents and denaturants on pFn and Fn, we tested their binding to gelatin (fig.1A,B) and to heparin (fig.1C,D) by two complementary protocols. The 'binding' assay measured the ability of detergent-pretreated protein to bind and be specifically eluted (as in table 1) by 4 M urea from a gelatin column (fig.1A) or by 2 M NaCl from a heparin column (fig.1C). In the 'elution' assay (fig.1B and D), untreated pFn was allowed to bind to the columns, then eluted directly by the denaturant. The latter assay is simpler in concept and practice and has been used more previously [15], but has some significant limitations: (i) Certain treatments (e.g., extensive reduction [20]) that are sufficient to prevent binding cannot detach the protein once it is bound and stabilized on an affinity column. (ii) Elution results from one or more of 3 experimentally unresolved causes: a change in pFn, in the gelatin/heparin, or in their binding interaction. (iii) One cannot detect reversible effects by 'renaturing' the protein before testing.

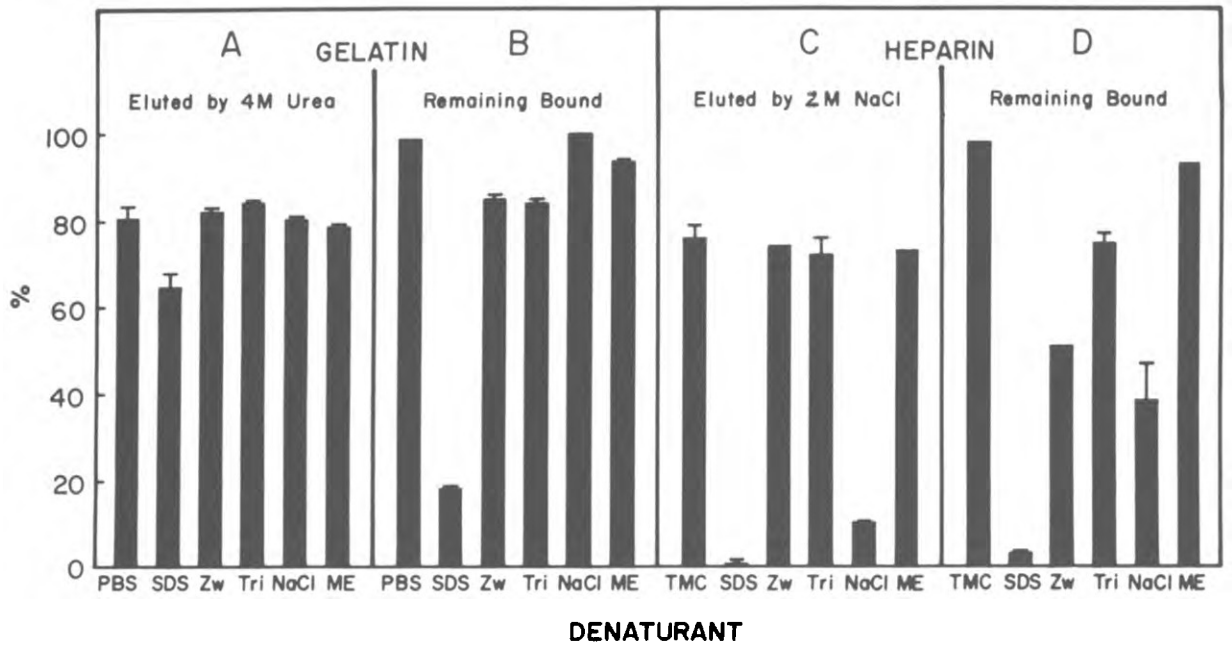

Fig.1. Effect of denaturants on gelatin- and heparin-binding of pFn. Affinity chromatography of 1-μl aliquots of ^{125}I-labeled pFn was performed on gelatin–Sepharose (A,B) or heparin–Sepharose (C,D). In the binding assays (A,C), the radioactivity eluted with 4 M urea (A) or 2 M NaCl (C) is expressed as percentage of total eluted with PBS + 1 M urea + 4 M urea (A) or TMC + 2 M NaCl + 5 M NaCl (C) (mean and upper range of duplicate experiments). Similar results (not shown) were obtained using Fn. In elution assays (B,D), the radioactivity remaining bound to the column is expressed as percentage of the total eluted by the denaturant or remaining bound (mean and upper range of duplicate experiments, except a single value for Zwittergent in D).

Control treatment with PBS (fig.1) did not affect gelatin binding (cf. table 1), and control heparin binding was in close quantitative agreement with earlier larger scale experiments [13]. Treatment (10 min) with the strong ionic detergent SDS diminished or destroyed binding activity by all 4 criteria (fig.1A–D). In contrast, NaCl affected only binding to heparin, confirming [13,15] the more highly ionic character of the latter interaction. Mild reduction with mercaptoethanol had no effect. (Authors in [20] have shown that more extensive reduction with dithiothreitol will prevent gelatin binding, but still cannot elute gelatin-bound Fn.) pFn and Fn were also not perturbed by the zwitterionic detergent Zwittergent 3-12, which is milder than ionic detergents [21], but does denature or inactivate some proteins [22–24], or by the nonionic detergents Triton X-100 [15] and Nonidet P-40 (not shown; similar throughout to Triton X-100). Comparison of fig.1A to B and C to D suggests these detergents may have a small effect, though, on the gelatin and heparin, or on their interaction with pFn.

4. DISCUSSION

The availability of a moderately strong detergent like Zwittergent which spares Fn's and pFn's ability to bind to gelatin and heparin will permit a number of interesting future experiments: (i) It has been reported [25] that fluorescently labeled pFn is taken up from the substratum into adhering cells. To understand the nature of this process, it is important to learn whether the endocytosed fluorescent material is intact pFn, or only degraded fragments; dissolution of the cells with Zwittergent would permit one to test it for gelatin- and heparin-binding activity. (ii) Cell-substratum adhesion sites contain a selected population of cellular proteins, including multiple pools of Fn [3,26]. Zwittergent selectively extracts some, but not all, of these proteins (unpublished); the present study

suggests it is a gentle reagent which may be useful in separating sub-populations of proteins involved to different degrees in the process of cell adhesion. (iii) The adhesion sites of cells grown on gelatin are particularly enriched in Fn [27]. Extraction of these sites with Zwittergent can be used to show whether the Fn is bound primarily to the gelatin (and so resistant to extraction), primarily within the cell, or whether there are multiple pools of the material (cf. [26]).

Fn and pFn have many biological activities [8,9], and correspondingly many binding affinities, for collagen, fibrin, proteoglycans, and other cellular components. The binding assays we have described here are simple, economical, and quantitative, and so will hopefully be useful in a variety of contexts.

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