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Heparin Enhances the Rate of Binding of Fibronectin to Collagen

Staffan JOHANSSON* and Magnus HÖÖK*
Department of Medical and Physiological Chemistry, University of Uppsala and Swedish University of Agricultural Sciences, The Biomedical Center, Box 575, S-751 23 Uppsala, Sweden

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^{125}I-labelled fibronectin is shown to bind to both native and denatured collagen immobilized on Sephadex beads in reactions that exhibit different kinetics. The rates of both reactions were enhanced by the presence of heparin or highly sulphated dextran sulphate but not by other glycosaminoglycans or dextran sulphates having low sulphate contents.

Fibronectin is a glycoprotein found in connective tissue and in blood plasma (for a review see Yamada & Olden, 1978). An important function of fibronectin is to serve as a link between collagenous structures and the surface of cells. Thus the attachment of certain cells to collagen substrata is mediated by fibronectin (Pearlstein, 1976; Klebe, 1974), as is the pinocytosis of denatured collagen by macrophages (Hopper *et al.*, 1976). Previous studies have shown that fibronectin binds preferentially to denatured rather than to native collagen (Jilek & Hörmann, 1978; Engvall *et al.*, 1978). In fact, conflicting results have appeared with regard to the binding of fibronectin to native collagen (Dessau *et al.*, 1978).

Recent reports have pointed to the possibility that glycosaminoglycans can affect the function of fibronectin. Thus (*a*) a fibronectin derivative made with a photoactivatable cross-linker preferentially cross-links to chondroitin sulphate when added to NIL cells grown in cell culture (Perkins *et al.*, 1979). (*b*) The extracellular matrix produced by fibroblasts grown *in vitro* contains in addition to collagen and fibronectin also heparan sulphate proteoglycans and hyaluronic acid (Hedman *et al.*, 1979). (*c*) The fibronectin-mediated phagocytosis of gelatin-coated test particles by liver slices is highly dependent on the presence of heparin (Saba *et al.*, 1978). (*d*) Addition of a suitable amount of heparin to a solution containing fibronectin or a mixture of fibronectin and collagen in both cases resulted in the formation of precipitates containing the protein(s) (Jilek & Hörmann, 1979). In view of these observations we decided to investigate the possible effect of glycosaminoglycans on the binding of fibronectin to collagen.

* Present address: Department of Biochemistry, University of Alabama Medical Center, University Station, Birmingham, AL 35294, U.S.A.

Materials

Fibronectin was isolated from human plasma as described by Johansson *et al.* (1979) and radiolabelled with ^{125}I by the chloramine-T method (Hunter, 1973). Analysis of the labelled protein by electrophoresis on polyacrylamide slab gels in the presence of sodium dodecyl sulphate followed by radioautography indicated the presence of one labelled component with the migration property corresponding to that of unlabelled plasma fibronectin. When subjected to affinity chromatography on gelatin–Sepharose more than 40% of the labelled material bound to the gel.

Neutral-salt-soluble collagen prepared from rat skin (Öbrink, 1972) was kindly given by Dr. K. Rubin and Dr. B. Öbrink at this institute. The preparation consisted mainly of type I collagen and was shown to be free of fibronectin (Rubin *et al.*, 1978). Collagen was denatured by incubation at 60°C for 60min. Ovalbumin was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

The glycosaminoglycans used have all been described in other communications from this laboratory (see Table 1). Dextran sulphates were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Methods

Coupling of proteins to Sephadex G-25

Native collagen, denatured collagen and ovalbumin (2.1mg of each) were each incubated with 100mg of CNBr-activated Sephadex G-25 (superfine grade) (Pharmacia Fine Chemicals) at 4°C (ovalbumin and native collagen) or 22°C (denatured collagen), essentially as described by Öbrink *et al.* (1975). Amino acid analysis of a sample of each gel indicated total contents of 0.3mg of denatured collagen, 0.3mg of native collagen and 0.5mg of ovalbumin per 100mg of gel. The

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protein-substituted gels were suspended in 10ml of phosphate-buffered saline (0.14M-NaCl/10mM-sodium phosphate buffer, pH 7.5) containing 0.2% bovine serum albumin and 0.02% NaN_3 (referred to as the 'stock suspension') and stored at 4°C.

Binding of ^{125}I-labelled fibronectin to collagen–Sephadex

Incubations were performed in a total volume of 1.1ml of phosphate-buffered saline containing 0.2% bovine serum albumin, 0.02% NaN_3 and 25μl of the stock suspension of the immobilized protein (corresponding to 750ng of protein, 1.5×10^5c.p.m. of ^{125}I-labelled fibronectin (corresponding to about 20ng of protein) and polysaccharides as indicated, in an end-over-end mixer, at room temperature (approx. 22°C) for the indicated periods of time. The incubation mixture was centrifuged at 2000*g* for 5min and the supernatant was removed. After the Sephadex beads had been washed three times with 2ml of phosphate-buffered saline, the amount of ^{125}I-labelled material associated with the beads was determined in an automatic γ-radiation counter (model 1195; Searle Analytic). Incubations were always performed in duplicate.

Incubation of collagen–Sephadex with proteinases

Protein-substituted Sephadex beads (25μl of the stock suspension) were incubated with 1mg of collagenase (type I; Sigma) in 1.1ml of 0.1M-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer, pH 7.6, containing 1.5% bovine serum albumin, 10mM-NaCl, 5mM-KCl and 5mM-$CaCl_2$ for 2h at 37°C, or with 1mg of crystalline trypsin in 1.1ml of phosphate-buffered saline for 1h at 37°C. The trypsin digestions were terminated by adding 1mg of soya-bean trypsin inhibitor. After the beads had been washed ^{125}I-labelled fibronectin (1.5×10^5 c.p.m.) was added and the mixture was incubated for 1h under the conditions of the assay procedure in the presence of 1μg of heparin/ml.

Results and Discussion

Binding of ^{125}I-labelled fibronectin to Sephadex beads substituted with native or denatured collagen

The time courses of the binding of ^{125}I-labelled fibronectin to Sephadex beads substituted with native collagen, denatured collagen and ovalbumin are shown in Fig. 1. Under the experimental conditions employed ^{125}I-labelled fibronectin readily bound to both types of collagen beads, although the binding of fibronectin to denatured collagen proceeded at a much higher rate than the binding to native collagen. Negligible amounts of ^{125}I radioactivity were recovered associated with the ovalbumin-substituted beads. The native collagen monomers were presumably not significantly denatured

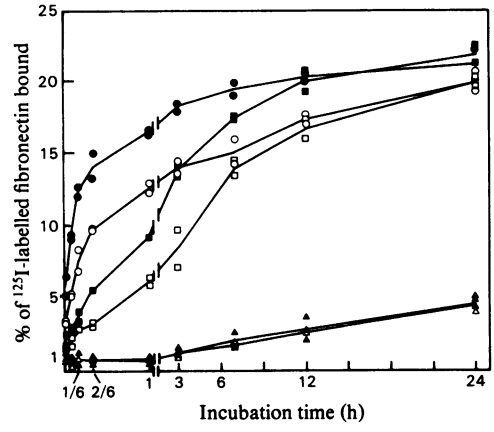

Fig. 1. *Time course of the binding of ^{125}I-labelled fibronectin to Sephadex beads substituted with ovalbumin, denaturated collagen and native collagen*

^{125}I-labelled fibronectin was incubated with Sephadex G-25 substituted with denatured collagen (○, ●), native collagen (□, ■) and ovalbumin (△, ▲) for the indicated periods of time (for further details see the Methods section). Black symbols indicate incubations performed in the presence of 1μg of heparin/ml.

during the incubation process, since preincubation for 24h of Sephadex substituted with native collagen before the addition of ^{125}I-labelled fibronectin did not alter the kinetics of the binding reaction.

To exclude the possibility that coupling of native collagen to Sephadex results in a denaturing of the protein the following approach was taken. A collagen monomer in its native triple-helical structure is largely resistent towards trypsin digestion (Piez, 1967), but the susceptibility increases on denaturation of the collagen monomer. Thus trypsin digestion would be expected to remove protein from the Sephadex substituted with denatured collagen but not from the Sephadex substituted with native collagen. Binding of ^{125}I-labelled fibronectin to trypsin-digested Sephadex beads substituted with denatured collagen was diminished to 53% compared with that bound to untreated beads. However, trypsin digestion did not affect the binding of ^{125}I-labelled fibronectin to Sephadex substituted with native collagen, indicating that the immobilized collagen monomers were not susceptible to the enzyme and had thus retained their native structure during the coupling procedures. Collagenase digestion of the beads decreased the amounts of fibronectin binding to both forms of immobilized collagen (to about 50% of that bound to undigested beads), indicating (*a*) that the collagen on the beads

was responsible for fibronectin binding and (*b*) that the collagen on the beads was accessible to enzymic digestion.

The findings described above suggest that fibronectin is capable of binding to native collagen but that this reaction exhibits different kinetics from the binding of fibronectin to denatured collagen. These results can at least to some extent explain the conflicting reports on the binding of fibronectin to native collagen, as different incubation conditions have been used in these studies.

Effect of sulphated polysaccharides on the collagen–fibronectin interaction

Heparin at a concentration of 1 μg/ml enhanced the rate of binding of ^{125}I-labelled fibronectin to Sephadex substituted with either native or denatured collagen (Fig. 1). The capacity of the two forms of immobilized collagen to bind ^{125}I-labelled fibronectin during a 24 h incubation was, however, not significantly changed by the presence of heparin (Fig. 1). The effect of various concentrations of heparin on the fibronectin–collagen interaction was investigated. Full effect was obtained at a polysaccharide concentration of 0.5 μg/ml for both forms of immobilized collagen. Increasing the concentration of heparin (up to 40 μg/ml) had no additional effect on the binding reaction (results not shown). The unspecific binding of ^{125}I-labelled fibronectin to immobilized ovalbumin was not affected by the presence of heparin (Fig. 1).

The specificity of the polysaccharide in the reaction was studied by incubating ^{125}I-labelled fibronectin with the two forms of immobilized collagen in the presence of various negatively charged polysaccharides (Table 1). Heparin preparations differing with regard to affinity for antithrombin (Höök *et al.*, 1976) were equally active in effecting the fibronectin–collagen interaction. All the other glycosaminoglycans tested, including hyaluronic acid, dermatan sulphate, chondroitin sulphate or heparan sulphates differing with regard to sulphate contents, were inactive in this respect. Dextran sulphate having a high sulphate content (preparation I in Table 1) was even more potent than heparin in enhancing the rate of the binding of fibronectin to collagen, whereas dextran sulphates having low sulphate contents were inactive. Since the active polysaccharides all have a high sulphate content, it appears likely that the charge density of a polysaccharide is of crucial importance for the effect of the polysaccharide to enhance the rate of binding of fibronectin–collagen interaction. The mechanisms whereby the anionic polysaccharides exert this effect are presently unknown.

Table 1. *Binding of ^{125}I-labelled fibronectin to immobilized collagen in the presence of negatively charged polysaccharides*

Sephadex beads substituted with native or denatured collagen were incubated for 1 h with 1.5×10^5 c.p.m. of ^{125}I-labelled fibronectin under assay conditions (see the Methods section) in the presence of the indicated polysaccharides at a concentration of 1 μg/ml (A) or 25 μg/ml (B). The amount of ^{125}I-labelled fibronectin binding to the beads in the absence of polysaccharide was set to 100%. The values represent the means of two incubations.

Polysaccharide added	Sulphate per disaccharide (mol/mol)	% of ^{125}I-labelled fibronectin bound to: Immobilized denaturated collagen (A)	(B)	Immobilized native collagen (A)	(B)
None	—	100		100	
Heparin (pig mucosa; Jacobsson *et al.*, 1979)	2.56	134	136	148	156
Heparin binding to antithrombin–Sepharose (HA-heparin; Höök *et al.*, 1976)		127	128	144	150
Heparin not binding to antithrombin–Sepharose (LA-heparin; Höök *et al.*, 1976)		128	129	144	143
Heparan sulphate (bovine liver; L. Kjellén, Å. Oldberg & M. Höök, unpublished work)	0.97	95	93	83	78
Heparan sulphate (pig mucosa; Jacobsson, 1979)	0.68	100	99	91	99
Heparan sulphate (human aorta; Jacobsson *et al.*, 1979)	0.50	98	95	91	99
Dermatan sulphate (pig mucosa; Jacobsson *et al.*, 1979)	1.18	91	87	84	81
Chondroitin sulphate (bovine cartilage; Jacobsson *et al.*, 1979)	0.61	98	108	91	83
Hyaluronic acid (rooster combs; Jacobsson *et al.*, 1979)	0	102	99	102	94
Dextran sulphate I	2.2	179		268	
Dextran sulphate II	0.28	106		103	
Dextran sulphate III	0.06	100		93	

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