Interaction of the small proteoglycan decorin with fibronectin

Involvement of the sequence NKISK of the core protein

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Decorin, an interstitial small proteoglycan, was shown to interact with fibronectin via its core protein. In a solid-phase assay, both high-affinity (K_D values between 10 and 20 nM) and low-affinity (K_D values between 110 and 130 nM) binding sites were found. The central position of decorin core protein is made up of several repeats containing NKISK in positions 85–89 and similar sequences in other repeats. The pentapeptide inhibited, albeit not completely, the high-affinity interaction between decorin and fibronectin in a specific charge-independent manner. Half-maximal inhibition occurred at a peptide concentration of 10 μ M. Core-protein-derived peptides that had been produced by endoproteinase Lys-C digestion were not inhibitory, but endoproteinase Arg-C-generated peptides served as inhibitors of binding. These results suggest that NKISK as a component of repetitive sequences of decorin is involved in the interaction between the proteoglycan and fibronectin.

INTRODUCTION

The extracellular matrix can be considered as a collection of macromolecules surrounding the cells and comprising a substrate to which the cells may be attached. The extracellular-matrix molecules themselves show multiple interactions with each other that individually may be rather weak but in their sum provide considerable strength. Fibronectin represents a prototype of a multifunctional adhesive glycoprotein that is capable of interacting with a variety of extracellular-matrix macromolecules and receptors in the plasma membranes [see Akiyama & Yamada (1986) Ruoslahti (1988a) and Mosher (1989) for reviews]. The pleiotropic functions of fibronectin are explained by the existence of functional domains, which can be isolated by limited proteolysis (Yamada *et al.*, 1980). Two heparin-binding domains with dissociation constants in the micromolar range and additional domains with lower affinity have been characterized (Richter *et al.*, 1981; Gold *et al.*, 1983; Sekiguchi *et al.*, 1983; Calaycay *et al.*, 1985; Skorstengard *et al.*, 1986; Ingham *et al.*, 1990). It had also been shown that isolated heparin-binding domains may interact at low ionic strength with dermatan sulphate, chondroitin sulphate and hyaluronan (Sekiguchi *et al.*, 1983). Interactions between intact fibronectin and hyaluronan and a chondroitin sulphate proteoglycan respectively at physiological ionic strength have also been reported (Lattera & Culp, 1982; Oldberg & Ruoslahti, 1982).

Fibronectin, however, is capable not only of binding glycosaminoglycans but additionally of reacting with specific proteoglycan core proteins. It had been shown that the core protein of the matrix-associated heparan sulphate proteoglycan binds to fibronectin (Heremans *et al.*, 1990). The core protein of the small dermatan sulphate proteoglycan decorin co-localized with fibronectin fibrils on the surface of cultured human fibroblasts. In an e.l.i.s.a. procedure decorin core protein bound to both high-affinity heparin-binding domains with similar efficiency to that of the intact proteoglycan (Schmidt *et al.*, 1987). Subsequent studies revealed also an interaction between decorin core protein and the cell-binding domain of fibronectin (Winnemöller *et al.*, 1991), which could explain the inhibitory effect of decorin on fibroblast

adhesion to a fibronectin substrate (Lewandowska *et al.*, 1987; Winnemöller *et al.*, 1991).

The secreted forms of human and bovine decorin are composed of 329 and 330 amino acid residues respectively (Krusius & Ruoslahti, 1986; Day *et al.*, 1987), a single glycosaminoglycan chain (Chopra *et al.*, 1985) and either two or three asparagine-bound oligosaccharides. A major portion of the core protein is composed of seven tandem repeats of a 24-amino acid-residue unit characterized by an arrangement of conserved leucine and asparagine residues (Ruoslahti, 1988b), but the number of reasonable tandem repeats may be increased to 12 (Fisher *et al.*, 1989). In the present paper we show that a pentapeptide sequence found in one of these repeats is involved in the interaction between decorin and fibronectin.

EXPERIMENTAL

Materials

Decorin was purified from the secretions of cultured human skin fibroblasts as previously described (Hauser *et al.*, 1989). Decorin core protein was obtained after treatment of decorin with chondroitin ABC lyase (Seikagaku Kogyo, Tokyo, Japan) as published previously (Glössl *et al.*, 1984). The decorin-derived pentapeptide NKISK was obtained from BTN Biotec, Nordhorn, Germany. The peptides QKTSK and LDNNKISR were kindly provided by Dr. B. Schmidt, Biochemie II, University of Göttingen, Göttingen, Germany. All peptides were purified on a Bakerbond wide-pore octadecyl column (4.6 mm $\times$ 359 mm; 5 μ m particle size; Baker, Phillipsburg, NJ, U.S.A.) with a linear gradient of 0.1% (v/v) trifluoroacetic acid in water to 0.1% (v/v) trifluoroacetic acid in 50% (v/v) acetonitrile at a flow rate of 0.5 ml/min. The gradient began after 10 min by increasing the concentration of acetonitrile at a rate of 0.5%/min until it approached 50%.

Fibronectin was prepared from outdated human plasma by chromatography on a gelatin-Sepharose 4B (Pharmacia-LKB, Freiburg, Germany) column as described by Ruoslahti *et al.* (1982). It was coupled to CNBr-activated Sepharose 4B (Phar-

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macia-LKB) according to the instructions of the manufacturer. BSA was immobilized analogously.

The radiochemicals L-[4,5- ^3H]leucine (2.6 TBq/mmol), L-[^{35}S]methionine (37 TBq/mmol) and $\text{Na}_2^{35}\text{SO}_4$ (carrier-free) were from Amersham-Buchler (Braunschweig, Germany), and the endoproteinases Lys-C and Arg-C were from Boehringer (Mannheim, Germany). Radioactively labelled decorin was obtained as described previously (Glössl *et al.*, 1984; Hausser *et al.*, 1989), and glycosaminoglycans were prepared therefrom by alkaline borohydride treatment.

Interaction between decorin and fibronectin

The interaction of fibronectin with decorin and decorin core protein was studied by a modification (Schmidt *et al.*, 1987) of the e.l.i.s.a. procedure of Engvall & Perlmann (1971). Monoclonal anti-decorin antibodies were replaced by affinity-purified rabbit polyclonal antibodies (Voss *et al.*, 1986), which were used at a final concentration of 0.17 μg of protein/ml. Goat anti-(rabbit IgG) antibody-peroxidase conjugate (Bio-Rad, Munich, Germany) was used as second antibody after 1:1000 dilution with 20 mM-sodium phosphate buffer, pH 7.4, containing 150 mM-NaCl, 0.1 % Tween 20 and 30 g of BSA/l. Colour development was with 1 mM-2,2'-azinobis-(3-ethylbenzthiazolinesulphonic acid) and 37 μM - H_2O_2 in 200 μl of 50 mM-sodium citrate buffer, pH 4.0. All assays were done at least in duplicate. In parallel incubations, colour development differed by less than 10 % when the absorbance at 410 nm was greater than 0.1.

Binding studies were complemented by an analysis of the interaction of [^3H]leucine-, [^{35}S]methionine- or [^{35}S]sulphate-labelled decorin with fibronectin under otherwise identical conditions. Bound ligand was quantitatively solubilized by incubating each micro-titre well for 2 h at 37 °C with 6000 U.S.P. units of papain (Merck, Darmstadt, Germany) in 200 μl of 0.1 M-sodium acetate buffer, pH 6.5, containing 5 mM-EDTA and 5 mM-cysteine.

When the interaction of fibronectin with proteolytically derived decorin fragments was to be studied, 5 μg of decorin core protein in 25 μl of 25 mM-Tris/HCl buffer, pH 8.5, containing 1 mM-EDTA (endoproteinase Lys-C) or 25 mM-ammonium bicarbonate buffer, pH 8.0 (endoproteinase Arg-C), was incubated with 1 μg of either enzyme for 16 h at 37 °C. The reaction was terminated by adding *N*-tosyl-L-lysylchloromethane to a final concentration of 5 mM, and the mixture was diluted with binding buffer to the desired core protein concentration.

Other methods

Decorin was quantified by hexuronic acid analysis (Bitter & Muir, 1962). From the data of decorin composition (Glössl *et al.*, 1984) it was calculated that 1 μmol of uronic acid is equivalent to about 700 μg of decorin core protein. The purity of the decorin preparations was tested, after treatment with chondroitin ABC lyase, by PAGE in the presence of SDS (Laemmli, 1970) followed by silver staining (Merril *et al.*, 1981). Protein bands migrating differently from decorin core protein, chondroitin ABC lyase and albumin could not be detected. The purity of [^{35}S]methionine-labelled decorin was assessed similarly by fluorography after SDS/PAGE. Scanning of the fluorogram revealed greater than 95 % purity. The purity of fibronectin was also determined by silver staining of the electrophoretically separated fibronectin stock solution and shown to be about 97 %.

RESULTS AND DISCUSSION

Decorin core protein interaction

Previous studies with monoclonal antibodies had shown that decorin and its glycosaminoglycan-free core protein bound to

immobilized fibronectin at pH 7.4 and at physiological ionic strength (Schmidt *et al.*, 1987). Since the antibodies differed in their reactivity towards intact and chondroitin ABC lyase-degraded decorin the stoichiometry of their binding has not been studied in sufficient detail. Use of [^{35}S]methionine-labelled decorin and decorin core protein showed unequivocally that both ligands bound to fibronectin in a quantitatively similar manner. When 2.5 pmol of either ligand was applied per micro-titre well, about 25 fmol was bound regardless of whether the proteoglycan had been degraded with chondroitin ABC lyase or not. A Scatchard diagram revealed the existence of both high-

Fig. 1. Scatchard analysis of decorin core protein binding to immobilized fibronectin

[^{35}S]Methionine-labelled decorin core protein was mixed with unlabelled core protein to give a specific radioactivity of 360 kBq/nmol. The ligand was incubated either in the absence (●) or in the presence (▲) of 250 μM -NKISK.

Fig. 2. Affinity chromatography of decorin (PG), its glycosaminoglycan-free core protein (Core) and its protein-free glycosaminoglycan chains (GAG) on fibronectin-Sepharose CL-4B

[^3H]Leucine-labelled decorin and its glycosaminoglycan-free core protein (10000 c.p.m. each) and [^{35}S]sulphate-labelled glycosaminoglycans derived from an equivalent amount of decorin by a β -elimination reaction (15000 c.p.m.) were applied to 300 μl columns of immobilized fibronectin. The column was eluted with 900 μl each of 0.1 M-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl (fraction 1), 0.5 M-NaCl (fraction 2) or 1 M-NaCl and 1 % (v/v) Triton X-100 (fraction 3).

Fig. 3. E.I.s.a. with fibronectin-coated wells and either decorin (● and ▲) or its glycosaminoglycan-free core protein (○ and □)

After coating, the wells were incubated with decorin (1.25 nmol of hexuronic acid/ml) or an equivalent amount of core protein in the presence of the indicated concentrations of either NKISK (● and ○) or QKTSK (▲ and △). (a) and (b) represent independent experiments with different doses of the peptide NKISK.

Fig. 4. Influence of decorin-derived peptides on the interaction between fibronectin and undegraded core protein

All fibronectin-coated wells were incubated with 0.6 μ g of undegraded decorin core protein/ml. Additions consisted of endo-proteinase Arg-C-generated peptides (▲, Endo Arg-C), endo-proteinase Lys-C-generated peptides (■, Endo Lys-C) or undegraded core protein (●, Core). Corrections were made for colour development in wells containing peptides only and no intact core protein.

affinity and low-affinity sites with K_D values of about 20 nM and 130 nM respectively for the intact proteoglycan and of 10 nM and 110 nM respectively for decorin core protein (Fig. 1).

Further evidence for the conclusion that it is the core protein and not the glycosaminoglycan chain that interacts with fibronectin was obtained by affinity chromatography on Sepharose-bound fibronectin. Only about 4% of free dermatan sulphate was retained by the affinity matrix, but more than 70% of intact proteoglycan or glycosaminoglycan-free core protein (Fig. 2).

Inhibition by the pentapeptide NKISK

Since lysine and arginine residues of decorin core protein are involved in receptor-mediated endocytosis of this proteoglycan

(Glössl *et al.*, 1983) we had considered the possibility that the repetitive sequences of the core protein, which are rich in basic amino acids, e.g. NKISK, could serve as binding sites for the endocytosis receptor. This did not appear to be the case, but the pentapeptide was able to inhibit the interaction between decorin and fibronectin (Figs. 1 and 3). Amino acids residues 85–89 of the mature core protein have the sequence NKISK, and the similar sequences NKISR and NKLTR are found in positions 201–205 and 249–253 respectively (Krusius & Ruoslahti, 1986). It should be noted from the data of Fig. 3(b) that complete inhibition of the interaction between decorin and fibronectin could not be achieved even at high peptide concentrations. Normally the inhibition was about 70% at a concentration of 0.25 mM (Fig. 3a), but occasionally, for reasons not yet understood, the inhibition amounted to only 25% at this peptide concentration. On the other hand, half-maximal inhibition occurred at a low peptide concentration (about 10 μ M). When increasing doses of decorin or core protein were incubated at a constant peptide concentration, the highest relative inhibition was observed at the lowest concentration of the macromolecules (result not shown). Further experiments showed that the effect of NKISK on decorin binding to fibronectin was independent of the incubation time. The relative inhibitory effect of NKISK remained unchanged between 15 min and 90 min of incubation. Control experiments indicated that the inhibitory action of NKISK was specific and not simply the consequence of its charge. QKTSK, the sequence of which is not found in decorin core protein, exhibited only a marginal influence on decorin binding to fibronectin (Fig. 3a). When the binding of [35 S]-methionine-labelled decorin core protein was investigated in the presence of a constant concentration of NKISK (250 μ M), the Scatchard diagram revealed only an increase of the K_D value for the interaction with the high-affinity site(s) (10 nM versus 21 nM), whereas the K_D value for an interaction with low-affinity site(s) remained unaltered (Fig. 1).

A further peptide LDNNKISRV, resembling amino acid sequences 198–206 (LDGNKISRV) and 247–255 (LDNNK-LTRV), was less inhibitory than NKISK and showed only about 50% of the effect of the latter peptide (result not shown).

Inhibition by decorin-derived peptides

The results described in the preceding section suggest that the sequence NKISK, which is present in one of the core protein

domains, is interacting with fibronectin. The weakly inhibitory peptide LDNNKISR_V contains an arginine residue in the penultimate position in place of a lysine residue in the more potent peptide. Since the tertiary structure of the core protein is not known, the structure of a peptide of optimal inhibitory activity could not be predicted with reasonable certainty. In an indirect approach to verify the importance of the sequence NKISK, core protein peptides were generated by digestion with either endoproteinase Lys-C or endoproteinase Arg-C and used as inhibitors of the interaction between core protein and fibronectin. The peptides produced by endoproteinase Lys-C digestion were indeed unable to compete for binding of decorin core protein to fibronectin. This was in contrast with the effect of peptides obtained by degradation with endoproteinase Arg-C (Fig. 4). In both digestions the complete disappearance of core protein had been verified by PAGE in the presence of SDS followed by silver staining. When [³⁵S]methionine-labelled core protein was used as ligand, competition for binding was also observed in the presence of endoproteinase Arg-C-derived peptides but not in the presence of endoproteinase Lys-C-derived peptides.

Taken together, the results described in this paper stress the importance of the core protein of decorin in the interaction of the proteoglycan with fibronectin and suggest the participation of amino acid residues 85–89 with the sequence NKISK in this process. The relative importance of several similar sequences that are present in the central position of the core protein could not be established in the present investigation. It was regularly found, however, that complete inhibition of the interactions between decorin and fibronectin could not be achieved by the pentapeptide, and hence the existence of other binding sites must be postulated. Further investigations on the decorin core protein domains responsible for binding probably require the production of mutagenized core proteins in prokaryotic or eukaryotic expression systems.

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