Decorin endocytosis: structural features of heparin and heparan sulphate oligosaccharides interfering with receptor binding and endocytosis

Heinz HAUSSER* and Hans KRESSE

Institute of Physiological Chemistry and Pathobiochemistry, University of Münster, D-48129 Münster, Germany

Receptor-mediated endocytosis of decorin depends on its core-protein-mediated interaction with a 51 kDa membrane protein, which, in addition to its core-protein-binding site, carries a binding site for glycosaminoglycan chains. Membrane-associated heparan sulphate as well as heparin are known to have an inhibitory effect on decorin endocytosis by cultured skin fibroblasts. In this study, structural features of both glycosaminoglycans required for binding to the 51 kDa protein and for inhibiting decorin endocytosis, were investigated. Upon digestion of [³H]glucosamine-labelled heparan sulphate with heparinase III, dodeca- and higher saccharides were able to interact with the receptor protein. In comparison with unbound fragments of the same size, bound fragments were enriched in N-sulphated disaccharides carrying one or two sulphate ester groups. Using heparinase III-generated fragments from [³⁵S]sulphate-labelled heparan sulphate chains, binding of fragments as small as

octasaccharides could be detected. Competition experiments between dermatan sulphate and chemically modified heparin revealed that N- and 6-O-sulphation of glucosamine residues are important structural elements for binding to the receptor, whereas iduronate-2-O-sulphate groups contribute to binding only to a limited extent. However, with respect to the inhibition of decorin endocytosis, 2-O-desulphation had a quantitatively similar effect to 6-O-desulphation. Furthermore, for maximal inhibition of decorin endocytosis, longer fragments were required than for binding to the receptor. Thus, it appears that heparin/heparan sulphate has to interact with additional component(s) for effective inhibition of decorin uptake.

Key words: core protein, dermatan sulphate, fibroblasts, heparinase, keratinocytes.

INTRODUCTION

Decorin is a prototype member of a growing family of small leucine-rich proteoglycans (see [1–3] for reviews). Structural hallmarks of these molecules are core proteins which are composed in their central part of leucine-rich repeat sequences and conserved cystine loops near the N- and C-terminal ends. Decorin of mammalian origin carries a single chondroitin/dermatan sulphate chain near the N-terminus, whereas in the closely related proteoglycan biglycan the core protein is most often substituted with two glycosaminoglycan chains. From the results of gene knock-out studies decorin is known to play an important role in the control of the lateral growth of type I collagen-containing fibrils [4]. It has also been suggested that it may participate in connecting type I and type VI collagen networks [5]. On the basis of the interactions with fibronectin [6] and thrombospondin [7], a modulating effect on cell adhesion has been proposed [8,9]. Recent investigations indicate that decorin may be involved in the regulation of the cell cycle via its binding to the epidermal growth factor receptor [10], and in the prevention of apoptosis [11]. Finally, the capability of decorin to form complexes with transforming growth factor- β [12] has led to successful trials of using decorin as a therapeutic agent in models of fibrotic diseases [13,14]. The related proteoglycan biglycan may have additional regulatory functions due to its capability to enter the nuclear compartment of at least some neuronal cells [15].

For performing such a variety of regulatory activities, control of the extracellular concentration of decorin (and biglycan) seems to be of great importance. Evidently, the amount of

extracellular decorin depends on the rates of biosynthesis and secretion on the one hand and on the rates of endocytosis and intralysosomal degradation on the other hand. Limited extracellular proteolysis of decorin core protein has been described [16,17], but degradation to monomeric constituents occurs only intralysosomally after cellular uptake. Fibroblasts and other cells of mesenchymal origin are known to efficiently internalize decorin by receptor-mediated endocytosis. Uptake is mediated by core protein–receptor interactions [18]. A 51 kDa protein present at the plasma membrane and in the endosomal compartment has been considered as the receptor [19]. The same protein has been shown to interact with biglycan, and both proteoglycans compete with each other for uptake by cultured fibroblasts [20]. In addition to the core protein binding site, which, at least in the case of decorin, interacts with structural elements of the leucine rich repeats in the central part of the core protein [21], the endocytosis receptor has a glycosaminoglycan binding site for heparin/heparan sulphate and dermatan sulphate. Interaction of heparin/heparan sulphate with the receptor protein leads to an inhibition of decorin endocytosis [22]. Thus, heparan sulphate proteoglycans present on the cell surface and/or within the extracellular matrix may modulate the rates of decorin and biglycan endocytosis, provided that they exhibit the structural features required for interaction with the decorin/biglycan endocytosis receptor [23].

Previous studies have indicated that endocytosed heparin, although interfering with decorin uptake, is not delivered to lysosomes, presumably because of the stability of the receptor–heparin complex at the acidic pH of the endosome. Instead, it is re-routed to the plasma membrane where it can be displaced by

Abbreviations used: GlcNSO₃, 2-deoxy-2-sulphamate-Glc; IdoA, L-iduronic acid.

* To whom correspondence should be addressed (e-mail kresse@uni-muenster.de), at Institut für Physiologische Chemie und Pathobiochemie, Waldeyerstrasse 15, D-48149 Münster, Germany.

exogeneously present heparin [22]. It could be shown additionally that heparan sulphate proteoglycans being present in the plasma membrane of certain cell types, e.g. in HaCaT keratinocytes, are able to block decorin endocytosis almost completely. Enzymic removal of membrane-associated heparan sulphate by heparinases was followed by a dramatic increase in the uptake rate of decorin. Only minor effects were observed after analogous treatment of fibroblasts [23].

In the present study we investigated the structural requirements of heparan sulphate and heparin respectively for binding to the receptor protein and for inhibition of decorin endocytosis by cultured fibroblasts. It will be shown that these requirements are not identical with regard to the minimal molecular size of inhibitory oligosaccharides and to their content of 2-O-sulphated iduronosyl residues.

EXPERIMENTAL

Cell culture and metabolic labelling

Human skin fibroblasts and human keratinocytes (HaCaT cell line) were maintained in culture as described [18]. For endocytosis experiments the concentration of NaHCO_3 was reduced from 2.2 g/l to 1.6 g/l to maintain a pH of 7.2 in an atmosphere of 95 % air/5 % CO_2 . This pH of the culture medium is necessary for normal intralysosomal catabolism of glycosaminoglycans.

To prepare [^{35}S]sulphate-labelled decorin from the culture supernatants of human skin fibroblasts, the cells were incubated in the presence of 0.37 MBq/ml of [^{35}S]sulphate (carrier free, approx. 1.6 TBq/mg S; ICN Biomedicals, Eschwege, Germany) for 72 h. For preparation of [^{35}S]sulphate- or [6- ^3H]GlcNAc-labelled heparan sulphate chains, keratinocytes were incubated either in the presence of 1.48 MBq/ml [^{35}S]sulphate or of 0.74 MBq/ml [^3H]GlcN (0.9–1.5 TBq/mmol; ICN Biomedicals) for 72 h. In all these incubations, fetal calf serum, which had been dialysed against 0.15 M NaCl, was added to a final concentration of 4 % (v/v). For metabolic labelling with [^{35}S]sulphate, streptomycin sesquisulphate was omitted.

Endocytosis

Endocytosis of [^{35}S]sulphate-labelled decorin was measured as described [18]. Endocytosis of decorin is followed by intralysosomal formation of inorganic sulphate, the major part of which is released into the culture medium. Therefore endocytosis is represented by the sum of the intracellular radioactivity and the ethanol-soluble radioactivity in the culture medium. For comparison, endocytosis is usually expressed as clearance rate, giving the volume of incubation medium (in microlitres) cleared from labelled decorin per hour and milligram of cell protein.

Preparation of proteoglycans and glycosaminoglycan chains

Decorin was prepared under non-denaturing conditions by precipitation with ammonium sulphate and subsequent anion-exchange chromatography on a Bio-Gel TSK DEAE-5PW HPLC column (Bio-Rad Laboratories, Munich, Germany) exactly as described [19]. Dermatan sulphate chains were obtained from bovine skin according to the procedure described by Fransson [24]. The crude glycosaminoglycan fraction was subjected to fractionated ethanol precipitation using 18 % and 36 % (v/v) ethanol.

For the preparation of heparan sulphate proteoglycans from the cell culture supernatants of keratinocytes, conditioned media (15 ml) were applied to a 0.5 ml column of DEAE-Trisacryl equilibrated with 20 mM Tris/HCl, pH 7.4, containing 150 mM

NaCl. After washing with 5 ml of this buffer, the proteoglycan fraction was eluted with 20 mM Tris/HCl, pH 7.4, containing 1 M NaCl. Peak fractions were pooled, made 0.1 % with BSA, dialysed against water and freeze-dried. The material was dissolved in 200 μl of 50 mM Tris/HCl buffer, pH 8.0, containing 60 mM sodium acetate and 50 mM NaCl, and incubated at 37 °C for 4 h with 100 m-units of chondroitinase ABC (Seikagaku Corporation, Tokyo, Japan) and 10 units of hyaluronidase (Sigma, Deisenhofen, Germany). The sample was then made 1 mM with CaCl_2 and digested overnight with Pronase (Sigma) at a concentration of 5 mg/ml. After 3-fold dilution with 20 mM Tris/HCl buffer, pH 7.4, the sample was again subjected to anion-exchange chromatography on a DEAE-Trisacryl column. The heparan sulphate chains eluted with 1 M NaCl were dialysed against $0.1 \times \text{PBS}$ and subsequently concentrated to one tenth of the eluate volume by freeze-drying.

Fragmentation and size fractionation of glycosaminoglycan chains

Enzymic depolymerization of heparan sulphate was performed either with heparinase I (Sigma) at a concentration of 2.5 units/ml or with heparinase III (heparitinase I from Seikagaku) at a concentration of 50 munits/ml, both in 100 mM sodium acetate, pH 7.0, containing 0.2 mM calcium acetate [25]. Samples were incubated for 16 h at 30 °C (heparinase I) and at 37 °C (heparinase III) respectively. Partial depolymerization of heparin with nitrous acid was performed as described [26]. Briefly, heparin was dissolved in 50 mM sodium nitrite at a concentration of 100 mg/ml, acidified to pH 1.5 with 6 M HCl and maintained at this pH for 4 min at room temperature. The resulting oligosaccharide mixture was neutralized with 1 M sodium bicarbonate and then precipitated with 4 vol. of ethanol containing 1.3 % potassium acetate.

Size fractionation of oligosaccharides was performed on a Superdex Peptide HR 10/30 column (Pharmacia Biotech, Freiburg, Germany) equilibrated and eluted with 0.5 M NH_4HCO_3 at a flow rate of 0.5 ml/min. Fractions of 150 μl were collected and small aliquots were taken for liquid-scintillation counting or determination of hexuronic acid. Estimates of the size of the fragments were based on the elution profiles of monosulphated dermatan sulphate disaccharide, trisulphated trisaccharide and a heparan sulphate dodecasaccharide fraction (provided by Dr. A. Schmidt, Institute of Arteriosclerosis Research, Münster, Germany).

Preparation of chemically modified heparin species

Unmodified heparin from porcine intestinal mucosa and partially N-desulphated N-reacetylated heparin were purchased from Sigma. Complete reacetylation was achieved by treating N-desulphated heparin, dissolved in 4.5 M sodium acetate containing 20 % (v/v) methanol, with acetic anhydride (five additions of 1/5 volume at 10 min intervals) at ambient temperature. Selective 2-O-desulphation was performed by freeze-drying an aqueous solution of the heparin sodium salt (4 mg/ml in water), brought to pH 12.5 by the addition of sodium hydroxide [27]. 6-O-Desulphated heparin, preferentially desulphated at the trisulphated disaccharide units, was obtained by treatment of the heparin pyridinium salt (dissolved in pyridine at a concentration of 10 mg/ml) with *N*-methyltrimethylsilyl-trifluoroacetamide (200 μl per ml of heparin solution; Sigma) for 2 h at temperatures between 65 °C and 100 °C [28]. After addition of an equal volume of 20 % (v/v) methanol, the reaction mixture was dialysed against running water. Then, 1 M NaOH was added to adjust the pH to 9–10. The mixture was dialysed again and finally lyo-

philized to recover the sodium salts of the various 6-O-desulphated heparin species.

Disaccharide analysis

The disaccharide composition of heparan sulphate and of chemically modified heparin species was analysed by strong anion-exchange chromatography after complete (more than 90%, as judged by gel chromatography) depolymerization to $\Delta 4,5$ -unsaturated disaccharides by exhaustive digestion with a mixture of heparinases I, II and III essentially as described [29]. The lyases were used at concentrations of 2.5 units/ml (heparinase I and heparinase II; both from Sigma) and 50 munits/ml (heparinase III; Seikagaku) respectively, and incubations were carried out in 100 mM sodium acetate, pH 7.0, containing 0.2 mM calcium acetate at 37 °C for 16 h. Disaccharides were recovered by chromatography on a Superdex Peptide column as described above. The disaccharide products were freeze-dried and subjected to SAX-HPLC on a Phenosphere 5 SAX 80 Å column (4.6 × 250 mm; Phenomenex, Hösbach, Germany). After the column had been equilibrated with the mobile phase (doubly deionized water adjusted to pH 3.5 with HCl) at 0.8 ml/min, samples were injected and disaccharides were eluted with a linear gradient of NaCl (0–0.75 M over 60 min) in the same mobile phase. Disaccharides were detected either by in-line monitoring of UV absorbance (A_{232} for unlabelled disaccharides) or by liquid-scintillation counting (after collection of 300 μ l fractions). Calibration was performed with a set of eight disaccharides provided by Professor J. T. Gallagher, CRC Department of Medical Oncology, Manchester, U.K.

Binding experiments

For binding experiments, the 51 kDa endocytosis receptor protein was purified from rat brain by subcellular fractionation and heparin affinity chromatography, exactly as described [30], and subjected to SDS/PAGE and Western blotting. After blocking the membranes with 3% BSA in 20 mM Tris/HCl, pH 7.4/150 mM NaCl, incubation with ligand was carried out in the same buffer containing 1% BSA (incubation buffer). Bound [35 S]sulphate-labelled ligand was visualized by autoradiography. For disaccharide analysis, blocking was performed with 0.1% Tween 20 in 20 mM Tris/HCl, pH 7.4/150 mM NaCl, and bound ligands were eluted by incubating the receptor-protein-containing membrane pieces with the same buffer containing 2 M NaCl. For competition experiments, individual lanes of the Western blots were incubated with biotinylated dermatan sulphate chains (10 μ g/ml) in the absence or presence of the different heparin species (2 μ g/ml). In this case, bound ligand was visualized by an enhanced chemiluminescent reaction (Amersham Pharmacia Biotech, Freiburg, Germany) after incubating the membrane strips with peroxidase-conjugated extravidin diluted 1:1000 in incubation buffer. Quantification was performed by densitometry using a Bio Image Whole Band Analyzer (MWG-Biotech, Ebersbach, Germany).

Other methods

Biotinylation of dermatan sulphate chains with D-biotin-*N*-hydroxysuccinimide ester (Roche Molecular Biochemicals, Mannheim, Germany) was performed according to the instructions of the manufacturer. Other methods were performed as cited earlier [19].

RESULTS

Heparin is a more potent inhibitor of decorin endocytosis than heparan sulphate

It had been shown before that membrane-associated heparan sulphate as well as heparin both have an inhibitory effect on decorin endocytosis [22,23]. Therefore, a direct comparison was made by studying the dose dependence of heparin and of mucosal heparan sulphate respectively on decorin endocytosis by cultured fibroblasts. It is evident from the results shown in Figure 1 that heparin is a more potent inhibitor of uptake than heparan sulphate. Fifty per cent of the maximally achievable inhibition required a heparin concentration of 2.5 μ g/ml and a heparan sulphate concentration of more than 500 μ g/ml respectively. Importantly, the maximal degree of inhibition that could be achieved was also higher for heparin than for heparan sulphate.

Heparinase III-resistant domains of heparan sulphate interact with the endocytosis receptor protein

The higher inhibitory activity of heparin suggested that sulphate-rich domains of heparan sulphate are required to promote the

Figure 1 Inhibition of decorin endocytosis by heparin and heparan sulphate

Skin fibroblasts were incubated for 3 h with 2×10^5 c.p.m. [35 S]Sulphate-labelled decorin in the presence of the indicated concentrations of heparin (●) and heparan sulphate (○) respectively.

Table 1 Disaccharide composition of heparin, mucosal heparan sulphate and heparan sulphate from HaCaT keratinocytes

Δ UA, 4-enehexuronic acid; GlcNSO₃, 2-deoxy-2-sulphamate-Glc; HS, heparan sulphate.

Disaccharide	Proportions of disaccharides		
	Heparin	Mucosal HS	HaCaT HS
Δ UA-GlcNAc	4	28	50
Δ UA-GlcNSO ₃	3	14	23
Δ UA-GlcNAc(6-OSO ₃)	5	12	8
Δ UA(2-OSO ₃)-GlcNAc	1	6	—
Δ UA-GlcNSO ₃ (6-OSO ₃)	12	8	3
Δ UA(2-OSO ₃)-GlcNSO ₃	6	15	12
Δ UA(2-OSO ₃)-GlcNAc(6-OSO ₃)	1	7	—
Δ UA(2-OSO ₃)-GlcNSO ₃ (6-OSO ₃)	67	5	4

Figure 2 Binding of heparan sulphate to the 51 kDa receptor protein

(A) Partially purified receptor protein (2 μ g per lane) was subjected to PAGE in the presence of SDS (total acrylamide concentration 10%) followed by Western blotting. Individual lanes were incubated with [35 S]sulphate-labelled heparan sulphate obtained from media of HaCaT cells (about 6×10^5 c.p.m./ml) before or after heparinase I and heparinase III digestion respectively. Bound material was visualized by radioautography. In (B), the elution profiles of the ligand preparations on a Superdex Peptide column are shown. 1, Undigested heparan sulphate; 2, heparinase III digest; 3, heparinase I digest. dp, degree of polymerization; V_0 , void volume; V_t , total volume.

inhibition of decorin endocytosis. For a direct investigation of the interaction between heparan sulphate and the endocytosis receptor, [35 S]sulphate- as well as [3 H]glucosamine-labelled heparan sulphate was prepared from the HaCaT keratinocyte cell line. The disaccharide composition of [3 H]glucosamine-labelled heparan sulphate indicated that the glycosaminoglycan

from this source has a lower degree of sulphation than the mucosal heparan sulphate (Table 1). By autoradiography, a direct interaction of the [35 S]sulphate-labelled material from keratinocytes with the endocytosis receptor protein could be shown after Western blotting of a partially purified receptor preparation (Figure 2A). Binding was not sensitive to treatment with heparinase III, which leaves the highly sulphated domains of the heparan sulphate chains intact. In contrast, almost no binding was observed after heparinase I digestion upon application of equal quantities of radioactivity.

Besides disaccharides, heparinase III digestion of heparan sulphate from HaCaT cells yields a series of oligosaccharides with degrees of polymerization of 4, 6, 8 and higher (Figures 3A and D). Of this material, only fragments with a degree of polymerization of 8 and higher were able to interact with the receptor (Figures 3C and F), whereas smaller fragments did not bind (Figures 3B and E). Using fragments from [3 H]glucosamine-labelled heparan sulphate chains, binding of oligosaccharides with a degree of polymerization less than 12 was barely detectable (Figure 3C), suggesting a high sulphate content and hence a high specific 35 S-radioactivity of these small interacting oligosaccharides. Maximum binding efficiency was observed for fragments with a degree of polymerization of approx. 18, which eluted in fractions 18–21. More than 85% of these fragments were bound to the receptor protein (Figures 3B and C). A comparison of the disaccharide compositions of bound and unbound [3 H]glucosamine-labelled dodeca- and tetradecasaccharides (which exhibited a more discriminatory binding behaviour with only 14% of the fragments being bound) revealed a lower content of unsulphated and monosulphated disaccharides in the binding oligosaccharides, whereas the N-sulphated disulphated and trisulphated disaccharides were enriched in this fraction (Table 2).

Importance of 6-O-sulphated and N-sulphated glucosamine residues for interaction with the endocytosis receptor

The disaccharide analysis of receptor-binding heparan sulphate fragments given in Table 2 does not necessarily provide the clue for the essential structural features of heparan sulphate required for binding to the receptor. The disaccharide composition of bound fragments may reflect the sum of essential elements and of non-essential but not interfering structures. Therefore, a series of chemically modified heparin species was prepared (Table 3) and tested for the ability to compete with the binding of dermatan sulphate to the glycosaminoglycan binding site of the receptor. Dermatan sulphate was chosen as ligand because it exhibits a different structure than heparin, but effective competition could nevertheless be anticipated [22]. Binding studies indicated that both N-sulphation and 6-O-sulphation of heparin are required to enable efficient competition with dermatan sulphate (Figure 4). Heparin species desulphated either only at the amino group or at the 6-O-position of glucosamine had almost completely lost their inhibitory activity. N-Desulphated and reacylated heparin even appeared to enhance slightly the binding of dermatan sulphate to the receptor. Desulphation of 2-O-sulphated iduronic acid residues yielded a heparin species with reduced inhibitory activity, which suggests that the presence of iduronate-2-O-sulphate residues contributes to the binding activity only to a certain extent.

Sulphation patterns for inhibition of decorin endocytosis and receptor binding are not identical

The chemically modified heparin species described in the preceding section were tested for their effect on decorin endocytosis

Figure 3 Size-distribution of heparinase III-resistant heparan sulphate fragments being bound to the receptor protein

[^3H]Glucosamine- (A–C) and [^{35}S]sulphate-labelled (D–F) heparan sulphate chains were prepared from the media of HaCaT cells, digested with heparinase III and used as ligand (3×10^5 c.p.m./ml in the case of [^3H]glucosamine-labelled material and 3.8×10^6 c.p.m./ml for the [^{35}S]sulphate-labelled fragments respectively) for purified receptor protein (approx. $10 \mu\text{g}$ per assay) electroblotted onto nitrocellulose membrane. Equal aliquots of the heparinase III digests (A and D), of the unbound (B and E) and of the bound (C and F) material were separated on a Superdex Peptide column. No binding was observed on nitrocellulose membrane without receptor protein. dp, degree of polymerization; V_0 , void volume; V_t , total volume.

Table 2 Disaccharide composition of bound and unbound [^3H]glucosamine-labelled heparan sulphate fragments (degree of polymerization 12–14) obtained by heparinase III-digestion

For analysis, fractions 26–29 shown in Figures 2(B) and 2(C) respectively were used. ΔUA , 4-enehexuronic acid; GlcNSO $_3$, 2-deoxy-2-sulphamate-Glc; HS, heparan sulphate.

Disaccharide	Proportion of disaccharides	
	Bound	Unbound
$\Delta\text{UA-GlcNAc}$	7	14
$\Delta\text{UA-GlcNSO}_3$	5	12
$\Delta\text{UA-GlcNAc(6-OSO}_3\text{)}$	6	11
$\Delta\text{UA(2-OSO}_3\text{)-GlcNAc}$	—	—
$\Delta\text{UA-GlcNSO}_3\text{(6-OSO}_3\text{)}$	18	12
$\Delta\text{UA(2-OSO}_3\text{)-GlcNSO}_3$	53	43
$\Delta\text{UA(2-OSO}_3\text{)-GlcNAc(6-OSO}_3\text{)}$	—	1
$\Delta\text{UA(2-OSO}_3\text{)-GlcNSO}_3\text{(6-OSO}_3\text{)}$	11	8

by cultured fibroblasts. The results shown in Figure 5 indicate that all modified heparin species, including the 2-O-desulphated one, were inhibitory to a similar degree, except for the heparin

with unsubstituted amino groups, which was without any effect. However, none of the modified heparins exhibited the same potency as untreated heparin.

The availability of heparin preparations differing in their degree of 6-O-desulphation enabled us to correlate desulphation and inhibitory activity. It can be deduced from the results shown in Table 4 that almost full inhibitory activity was retained when the content of 6-O-sulphated glucosamine residues was reduced by 70%.

Treatment of heparan sulphate with heparinase III has different effects on receptor binding and on decorin endocytosis

The non-identity of the sulphation patterns required for interaction with the receptor protein and for inhibition of decorin endocytosis respectively may suggest that additional interactions of heparin/heparan sulphate chains are necessary for their inhibitory activity. A comparison was therefore made of the inhibitory effect of intact heparan sulphate chains and of chains digested with heparinase III. The results summarized in Table 5 demonstrate that heparinase III-treated heparan sulphate chains are less inhibitory of decorin endocytosis than intact chains. This contrasts with the finding of an unimpaired interaction of heparan

Table 3 Disaccharide compositions of the chemically modified heparin species

NdS, N-desulphated heparin; NdS-NAc, N-desulphated N-reacetylated heparin; 2-O-dS, 2-O-desulphated heparin; 6-O-dS, 6-O-desulphated heparin; ΔUA, 4-enehexuronic acid; GlcNSO₃, 2-deoxy-2-sulphamate-Glc; HS, heparan sulphate.

Disaccharide	Proportions of disaccharides						
	Heparin	NdS	NdS-NAc	2-O-dS	6-O-dS (65 °C)*	6-O-dS (80 °C)*	6-O-dS (100 °C)*
ΔUA-GlcNAc	4	7	8	6	11	12	15
ΔUA-GlcNSO ₃	3	—	—	12	4	5	22
ΔUA-GlcNAc(6-OSO ₃)	5	4	20	5	1	1	2
ΔUA(2-OSO ₃)-GlcNAc	1	3	10	—	2	2	2
ΔUA(2-OSO ₃)-GlcNH ₂ (6-O-SO ₃)	—	52	1	—	—	—	—
ΔUA-GlcNSO ₃ (6-OSO ₃)	12	—	—	70	1	9	6
ΔUA(2-OSO ₃)-GlcNSO ₃	6	—	—	—	30	41	43
ΔUA(2-OSO ₃)-GlcNAc(6-OSO ₃)	1	18	61	—	1	2	8
ΔUA(2-OSO ₃)-GlcNSO ₃ (6-OSO ₃)	67	—	—	3	38	21	1

* Temperature during desulphation.

Figure 4 Competition of chemically modified heparin species for binding of dermatan sulphate to the 51 kDa receptor protein

Partially purified receptor protein (about 2 μg/lane) was subjected to PAGE in the presence of SDS and Western blotting, followed by incubation of individual lanes with biotinylated dermatan sulphate chains (10 μg/ml) in the absence or presence of the heparin species indicated (2 μg/ml). The 6-O-desulphated (6-O-dS) heparin used was obtained at a reaction temperature of 100 °C. Bound dermatan sulphate chains were quantified by a chemoluminescence reaction. The assays were done in triplicate and the range of results is given. 2-O-dS, 2-O-desulphated species; NdS, *N*-desulphated heparin; NdS-NAc, *N*-desulphated *N*-reacetylated heparin.

sulphate with the receptor protein upon digestion with heparinase III (Figure 2).

Heparin fragments that inhibit decorin uptake best are larger than those required for binding to the receptor

Owing to the clustered organization of sulphate-rich domains on heparan sulphate chains and their low inhibitory activity on decorin uptake, heparan sulphate is not suited to determine the optimal chain-length for the inhibition of decorin uptake. Therefore, heparin was subjected to limited nitrous acid degradation and the products were size fractionated (Figure 6, upper panel). In comparison with the profiles shown in Figure 3, the elution positions of fragments of an identical degree of polymerization

Figure 5 Inhibition of decorin endocytosis by chemically modified heparin species

Fibroblasts were incubated with 2 × 10⁵ c.p.m./ml of [³⁵S]sulphate-labelled decorin in the absence or presence of the indicated heparin species (13.5 nmol hexuronic acid/ml). The 6-O-desulphated (6-O-dS) heparin was obtained at a reaction temperature of 100 °C. The assays were done in triplicate. Mean values ± S.E.M. are given. 2-O-dS, 2-O-desulphated species; NdS, *N*-desulphated heparin; NdS-NAc, *N*-desulphated *N*-reacetylated heparin.

Table 4 Inhibition of decorin endocytosis by heparin species varying in their degree of 6-O-desulphation

Skin fibroblasts were incubated with [³⁵S]sulphate-labelled decorin (2 × 10⁵ c.p.m./ml) for 3 h in the presence of heparin (13.5 nmol hexuronic acid/ml), 6-O-desulphated at the temperature indicated. The clearance rate in the heparin-free control was 19.7 ± 2.6 μl · h⁻¹ · (mg of cell protein)⁻¹. Mean values of two independent experiments are given.

	Percentage of 6-O-sulphate groups remaining in		Endocytosis (% of control)
	Trisulphated disaccharide	All disaccharides	
Untreated	100	100	33.8 ± 3.0
65 °C	57	48	35.6 ± 3.8
80 °C	31	39	39.5 ± 2.8
100 °C	1	20	62.4 ± 0.5

Table 5 Influence of heparinase III-treatment of kidney heparan sulphate on the inhibition of decorin uptake

Skin fibroblasts were incubated with [35 S]sulphate-labelled decorin (200 000 c.p.m./ml) for 3 h in the absence or presence of the inhibitors indicated (67.5 nmol hexuronic acid/ml). The incubations were done in triplicate, and mean values $\pm$ S.D. are given.

Inhibitor	Clearance [$\mu\text{l} \cdot \text{h}^{-1} \cdot (\text{mg of cell protein}^{-1})$]	P value
None	108.3 $\pm$ 5.7	
Heparan sulphate	84.9 $\pm$ 1.8	
Heparinase III-digested heparan sulphate	95.2 $\pm$ 4.0	0.031*; 0.016†
Heparin	38.2 $\pm$ 1.6	

* Compared with the uninhibited endocytosis.

† Compared with the endocytosis in the presence of undigested heparan sulphate.

Figure 6 Influence of the size of heparin fragments on their inhibitory effect on decorin endocytosis

Heparin was partially degraded by nitrous acid treatment as described in the Materials and methods section. In (A), the elution profile of the reaction products on a Superdex Peptide column is shown. Fractions were pooled as indicated by the bars. dp, degree of polymerization; V_0 , void volume; V_t , total volume. In (B), endocytosis of [35 S]sulphate-labelled decorin (2×10^5 c.p.m./ml) was determined in the absence or presence of the heparin fractions indicated (13.5 nmol hexuronic acid/ml). C, control, no saccharide added; H, undegraded heparin.

are shifted to lower fraction numbers due to the higher sulphate content of heparin-derived oligosaccharides. When equal quantities of hexuronic acids of the degradation products were tested for their inhibitory effect on decorin endocytosis, it became evident that significant inhibition could only be achieved with fractions containing deca- and higher saccharides (Figure 6, lower panel).

DISCUSSION

The results provided in the present study add the decorin/biglycan endocytosis receptor to the extensive list of proteins interacting with heparan sulphate and heparin (see [31–35] for recent reviews). The heparan sulphate moiety of various proteoheparan sulphate species has been shown to serve as a scaffold structure for quite a diverse set of proteins. Proteins interacting

with heparan sulphate include components of the extracellular matrix, lipoproteins, members of different families of cytokines, cytokine receptors, cell adhesion molecules, matrix receptors, enzymes, and possibly also nuclear regulatory proteins. On this basis heparan sulphate proteoglycans have been shown to be critically involved in complex biological phenomena, including organogenesis during embryonic development, control of cell growth, tumour metastasis, leukocyte trafficking and angiogenesis.

Heparan sulphate chains are characterized by the presence of oligosaccharide domains differing in the extent of N-acetylation/N-sulphation, the degree of GlcA C-5 epimerization and the number and position of ester sulphate groups. A typical heparan sulphate chain is composed of three kinds of domains. One domain consists predominantly of unsulphated GlcA-GlcNAc disaccharide units. A second domain is typically composed of alternating GlcA-GlcNAc and IdoA-GlcNSO₃ disaccharide units with a variable proportion of 6-O-sulphated GlcN residues, where IdoA is L-iduronic acid and GlcNSO₃ is 2-deoxy-2-sulphamate-Glc. Finally, there are clusters rich in IdoA(2-O-SO₃)-GlcNSO₃(6-O-SO₃) disaccharides [34]. In rare cases, unique protein-binding heparan sulphate sequences have been identified. The antithrombin-binding region in heparin and heparan sulphate contains an O-sulphate group at C-3 of a single GlcNSO₃ residue, and N-unsubstituted GlcN residues are enriched in sequences interacting with L- and P-selectin [36]. However, in most cases, common structural features of heparan sulphate are sufficient for protein binding. In the well-studied case of interaction with fibroblast growth factor-2, a hexasaccharide composed of IdoA(2-O-SO₃)-GlcNSO₃ disaccharide units is required, but the presence of 6-O-sulphated GlcN residues is tolerated. The latter residues may be essential for other interactions, e.g. for the binding to platelet-derived growth factor [37] and lipoprotein lipase [38].

Common heparan sulphate structures seem also to be responsible for the interaction with the decorin/biglycan endocytosis receptor. For a discussion of these structural features it is mandatory to differentiate between structures being directly involved in binding to the 51 kDa receptor protein and structures needed for interfering with receptor-mediated endocytosis of the two small proteoglycans. From the analysis of receptor-binding heparan sulphate oligosaccharides prepared from HaCaT cells it appeared that such an oligosaccharide had to consist of at least four disaccharide units. However, only a very limited number of octasaccharides appear to possess the essential structural features for binding, as only about 0.1 % of the [3 H]glucosamine-labelled material of this size was bound to the receptor. This low binding efficiency apparently was not due to limited binding capacity, as judged from the almost complete binding (> 85 %) of fragments with a degree of polymerization of approx. 18. With increasing fragment size, the probability for possessing a structure suited for binding increases, indicated by the increasing fraction of binding oligosaccharides with a degree of polymerization of 10 (2 % bound), 12–14 (14 % bound) and approx. 18 (> 85 % bound). Binding efficiency decreases for very large fragments, possibly due to steric hindrance in the solid-phase binding assay used in this study. From the disaccharide composition of receptor-binding heparan sulphate dodecasaccharides, it can be deduced that an average of five out of six glucosamine residues will be N-sulphated, two of the glucosamine residues will carry a 6-O-sulphate group, and four of the uronic acid residues will be 2-O-sulphated. The highest relative enrichment in comparison with unbound fragments of the same size was found for the two disaccharides carrying N- and 6-O-sulphate groups simultaneously. Competition studies between dermatan sulphate and

chemically modified heparin corroborated the importance of 6-O-sulphated and N-sulphated glucosamine residues for receptor binding.

Interaction between the receptor protein and heparan sulphate/heparin is likely to be a prerequisite for inhibition of decorin endocytosis. However, the inhibition of endocytosis by modified heparin fragments provides evidence for the conclusion that the latter effect requires additional structural features. Neither N-sulphation nor 2-O- and 6-O-sulphation respectively were absolutely essential for inhibitory activity, but they contributed to it. In addition, larger saccharides than the ones needed for receptor binding exhibited the greatest potency. Thus, whereas heparan sulphate-derived fragments with a degree of polymerization of approx. 18 were almost completely bound by immobilized receptor protein, longer fragments were required for maximal inhibitory activity, even though these oligosaccharides were derived from heparin, where, due to the more uniform structure, appropriate binding sites are expected on shorter fragments. This suggests that maximal inhibition requires the interaction of heparin/heparan sulphate with two proteins, analogously for example to the binding to fibroblast growth factor-2 and its receptor [39]. The second partner(s) to which heparin/heparan sulphate should bind for influencing the rate of decorin uptake remain(s) to be defined. Candidate proteins are cell-associated matrix proteins like collagens, fibronectin and thrombospondin, which are known to interact with heparin and heparan sulphate and which may negatively influence the transfer of the receptor from the cell surface to the endosome. A different mode of action can also be envisaged: interference with the uncoupling of decorin from its receptor, which would then lead to the re-routing of decorin to the cell surface. Such re-routing of endocytosed heparin had been shown before [22].

The proposal of a dual interaction of heparin/heparan sulphate during decorin endocytosis raises the question as to whether a single glycosaminoglycan chain may harbour both binding sites. This is certainly true for heparin. However, the dose dependence of the inhibition of decorin endocytosis by mucosal heparan sulphate suggests that only some, but not all, chains possess the appropriate structures for interacting simultaneously with the receptor and the not yet defined additional component(s). From the data on the dose-dependent inhibition of decorin endocytosis by exogenously added soluble heparan sulphate chains (Figure 1), it appears that some chains might interact only with either the receptor or the additional binding partner(s). Such a model would best explain the failure to achieve complete inhibition of decorin endocytosis. For membrane-bound heparan sulphate proteoglycans, a greater efficiency of inhibition of endocytosis can be envisaged, because the receptor and the additional component(s) could independently interact with one of several chains linked to the same core protein. That this may indeed be the case may be deduced from the fact that membrane-bound heparan sulphate from HaCaT cells, which has an even lower sulphation degree than mucosal heparan sulphate (Table 1), is nevertheless strongly inhibitory when HaCaT cells are challenged with decorin [23].

The efficient interaction of highly sulphated heparan sulphate domains with the decorin/biglycan endocytosis receptor opens the possibility of a dual function of this protein. In skin fibroblasts and other mesenchymal cells its main function may indeed be that of an endocytosis receptor for decorin and biglycan. However, in cells containing sulphate-rich heparan sulphate either on their cell surface or in their pericellular matrix, e.g. in certain epithelial cells, the 51 kDa protein could become immobilized on the cell surface by interacting with such heparan sulphate species and could then serve as an integrin-independent

mediator of cell-matrix interactions. One function could simply be the interaction with extracellular heparan sulphate. Furthermore, the 51 kDa protein could interact simultaneously with both small proteoglycans and heparan sulphate, thereby mediating the association of small proteoglycans with the cell surface. For example, it is known that suprabasal keratinocytes are surrounded by biglycan [40,41]. The mechanism responsible for this association has so far not been identified. Also, a supra-structural complex could be formed at the cell surface, consisting of a heparan sulphate proteoglycan, the receptor and small proteoglycan bound to an additional component of the extracellular matrix. Such a matrix-receptor function of the 51 kDa protein could be modulated by changes in the fine structure of the heparan sulphate on and in the vicinity of the cells, as occurs, for example, during differentiation.

We are very much indebted to Professor J. T. Gallagher for helpful suggestions and for the generous gift of reference disaccharides. Furthermore, we thank Dr. A. Schmidt for providing heparan sulphate-derived oligosaccharides. The expert technical help of Ms B. Liel and Mr. S. Budny is gratefully acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft, SFB 310, Project B2.

REFERENCES

- 1 Iozzo, R. V. (1998) *Annu. Rev. Biochem.* **67**, 609–652
- 2 Hocking, A. M., Shinomura, T. and McQuillan, D. J. (1998) *Matrix Biol.* **17**, 1–19
- 3 Kresse, H., Hausser, H. and Schönherr, E. (1993) *Experientia* **49**, 403–416
- 4 Danielson, K. G., Bribault, H., Holmes, D. F., Graham, H., Kadler, K. E. and Iozzo, R. V. (1997) *J. Cell Biol.* **136**, 729–743
- 5 Keene, D. R., Ridgway, C. C. and Iozzo, R. V. (1998) *J. Histochem. Cytochem.* **46**, 215–220
- 6 Schmidt, G., Robenek, H., Harrach, B., Glössl, J., Nolte, V., Hörmann, H., Richter, H. and Kresse, H. (1987) *J. Cell Biol.* **104**, 1683–1691
- 7 Winnemöller, M., Schmidt, G. and Kresse, H. (1991) *Eur. J. Cell Biol.* **54**, 10–17
- 8 Winnemöller, M., Schön, P., Vischer, P. and Kresse, H. (1992) *Eur. J. Cell Biol.* **59**, 47–55
- 9 Merle, B., Malaval, L., Lawler, J., Delmas, P. and Clezardin, P. (1997) *J. Cell. Biochem.* **67**, 75–83
- 10 Moscatello, D. K., Santra, M., Mann, D. M., McQuillan, D. J., Wong, A. J. and Iozzo, R. V. (1998) *J. Clin. Invest.* **101**, 406–412
- 11 Schönherr, E., O'Connell, B. C., Schittny, J., Robenek, H., Fastermann, D., Fisher, L. W., Plenz, G., Vischer, P., Young, M. F. and Kresse, H. (1999) *Eur. J. Cell Biol.* **78**, 44–55
- 12 Yamaguchi, Y., Mann, D. M. and Ruoslahti, E. (1990) *Nature (London)* **346**, 281–284
- 13 Border, W. A., Noble, N. A., Yamamoto, T., Harper, J. R., Yamaguchi, Y., Pierschbacher, M. D. and Ruoslahti, E. (1992) *Nature (London)* **360**, 361–364
- 14 Isaka, Y., Brees, D. K., Ikegaya, K., Kaneda, Y., Imai, E., Noble, N. A. and Border, W. A. (1996) *Nature Med.* **2**, 418–423
- 15 Liang, Y., Haring, M., Roughley, P. J., Margolis, R. K. and Margolis, R. U. (1997) *J. Cell Biol.* **139**, 851–864
- 16 Witsch-Prehm, P., Miehke, R. and Kresse, H. (1992) *Arthritis Rheum.* **35**, 1042–1052
- 17 Imai, K., Hiramatsu, A., Fukushima, D., Pierschbacher, M. D. and Okada, Y. (1997) *Biochem. J.* **322**, 809–814
- 18 Glössl, J., Schubert-Prinz, R., Gregory, J. D., Damle, S. P., von Figura, K. and Kresse, H. (1983) *Biochem. J.* **215**, 295–301
- 19 Hausser, H., Hoppe, W., Rauch, U. and Kresse, H. (1989) *Biochem. J.* **263**, 137–142
- 20 Hausser, H., Ober, B., Quentin-Hoffmann, E., Schmidt, B. and Kresse, H. (1992) *J. Biol. Chem.* **267**, 11559–11564
- 21 Hausser, H., Schönherr, E., Müller, M., Liszio, C., Bin, Z., Fisher, L. W. and Kresse, H. (1998) *Arch. Biochem. Biophys.* **349**, 363–370
- 22 Hausser, H. and Kresse, H. (1991) *J. Cell Biol.* **114**, 45–52
- 23 Hausser, H., Witt, O. and Kresse, H. (1993) *Exp. Cell Res.* **208**, 398–406
- 24 Fransson, L.-Å. (1968) *Biochim. Biophys. Acta* **156**, 311–316
- 25 Turnbull, J. E. and Gallagher, J. T. (1991) *Biochem. J.* **273**, 553–559
- 26 Ishihara, M., Tyrrell, D. J., Stauber, G. B., Brown, S. and Cousens, L. S. (1993) *J. Biol. Chem.* **268**, 4675–4683
- 27 Jaseja, M., Rej, R. N., Sauriol, F. and Perlin, A. S. (1989) *Can. J. Chem.* **67**, 1449
- 28 Ishihara, M., Takano, R., Kanda, T., Hayashi, K., Hara, S., Kikuchi, H. and Yoshida, K. (1995) *J. Biochem.* **118**, 1255–1260

-
- 29 Sanderson, R. D., Turnbull, J. E., Gallagher, J. T. and Lander, A. D. (1994) *J. Biol. Chem.* **269**, 13100–13106
- 30 Hausser, H., Wedekind, P., Sperber, T., Peters, R., Hasilik, A. and Kresse, H. (1996) *Eur. J. Cell Biol.* **71**, 325–331
- 31 Vlodavsky, I., Miao, H. Q., Medalion, B., Danagher, P. and Ron, D. (1996) *Cancer Metastasis Rev.* **15**, 177–186
- 32 Gallagher, J. T. (1997) *Biochem. Soc. Trans.* **25**, 1206–1209
- 33 Stringer, S.E. and Gallagher, J. T. (1997) *Int. J. Biochem. Cell Biol.* **29**, 709–714
- 34 Lindahl, U., Kusche-Gullberg, M. and Kjellén, L. (1998) *J. Biol. Chem.* **273**, 24979–24982
- 35 Tanaka, Y., Kimata, K., Adams, D. H. and Eto, S. (1998) *Proc. Assoc. Am. Physicians* **110**, 118–125
- 36 Koenig, A., Norgard-Sumniht, K., Linhardt, R. and Varki, A. (1998) *J. Clin. Invest.* **101**, 877–889
- 37 Feyzi, E., Lustig, F., Fager, F., Spillmann, D., Lindahl, U. and Salmivirta, M. (1997) *J. Biol. Chem.* **272**, 5518–5524
- 38 Parthasarathy, N., Goldberg, I. J., Sivaram, P., Mulloy, B., Flory, D. M. and Wagner, W.D. (1994) *J. Biol. Chem.* **269**, 22391–22396
- 39 Guimond, S., Maccarana, M., Olwin, B. B., Lindahl, U. and Rapraeger, A. C. (1993) *J. Biol. Chem.* **268**, 23906–23914
- 40 Bianco, P., Fisher, L. W., Young, M. F., Termine, J. D. and Robey, P. G. (1990) *J. Histochem. Cytochem.* **38**, 1549–1563
- 41 Schönherr, E., Beavan, L. A., Hausser, H., Kresse, H. and Culp, L. A. (1993) *Biochem. J.* **290**, 893–899
-

Received 9 August 1999/27 September 1999; accepted 2 October 1999