

Binding of Heparin and of the Small Proteoglycan Decorin to the Same Endocytosis Receptor Proteins Leads to Different Metabolic Consequences

Heinz Hausser and Hans Kresse

Institute of Physiological Chemistry and Pathobiochemistry, University of Münster, D-4400 Münster, Germany

Abstract. Decorin, a small interstitial dermatan sulfate proteoglycan, is turned over in cultured cells of mesenchymal origin by receptor-mediated endocytosis followed by intralysosomal degradation. Two endosomal proteins of 51 and 26 kD have been implicated in the endocytotic process because of their interaction with decorin core protein. However, heparin and protein-free dermatan sulfate were able to inhibit endocytosis of decorin in a concentration-dependent manner. After Western blotting of endosomal proteins,

there was competition for binding to the 51- and 26-kD proteins between heparin and decorin. In spite of its high-affinity binding, heparin was poorly cleared from the medium of cultured cells and then catabolized in lysosomes. In contrast to decorin, binding of heparin to the 51- and 26-kD proteins was insensitive to acidic pH, thus presumably preventing its dissociation from the receptor in the endosome. Recycling of heparin to the cell surface after internalization could indeed be demonstrated.

DECORIN (small dermatan sulfate proteoglycan II) is a member of the family of small interstitial proteoglycans (14, 28). It has been found in all tissues so far investigated (43). The mature molecule consists of a core protein with a relative molecular mass of 36,319 as deduced from cloned cDNA (18), a single glycosaminoglycan chain linked to the serine residue at position 4 (4) and either two or three asparagine-bound oligosaccharides (9). The central part of the core protein is made up by 10 leucine-rich repeats. Similar repeats have been found in several other proteins, for example in the leucine-rich α_2 -glycoprotein of human serum (27, 38) the platelet glycoprotein Ib (24), the human carboxypeptidase N high molecular weight subunit (39), and have been implicated in protein-protein interactions.

Interactions of decorin with several components of the extracellular matrix have been demonstrated. Through its core protein it binds to types I and II collagen (33, 34, 35), thereby affecting fibril formation (13, 42). Decorin core protein is also responsible for an interaction with fibronectin (32) which may lead to an inhibition of fibroblast adhesion to fibronectin substrates (21, 44). Recently, a role of decorin in the control of cell proliferation has been suggested (46), possibly by its ability to bind to transforming growth factor- β (45).

Therefore, the regulation of the extracellular concentration of decorin by the rates of its biosynthesis and of its degradation seems to be of great physiological importance. It has been shown that under tissue culture conditions a significant portion of secreted decorin is recaptured by the cells and degraded in the lysosomes (31). The protein moiety of decorin is responsible for its uptake by receptor-mediated endocytosis (27, 40).

Lysine and arginine residues have been shown to be important for the uptake properties (8), although the structure of the receptor-binding domain of the core protein is not yet known. Endosomal proteins of 51 and 26 kD have been shown to have decorin-binding properties and were considered as putative components of the decorin endocytosis receptor on the basis of the following additional findings (12). They co-precipitated with a proteoglycan core protein-antibody complex and showed strong binding to immobilized dermatan sulfate proteoglycan. Furthermore, they were sensitive to trypsin treatment of intact cells suggesting their presence on the cell membrane. In this report it will be shown that heparin and some other highly sulfated glycosaminoglycans are also interacting with these proteins and may serve as inhibitors of decorin uptake by cultured fibroblasts. In spite of the high affinity binding of heparin, however, its rate of endocytosis is at least a magnitude lower than that of decorin which might be explained by the stability of the heparin-receptor complex at acid pH.

Materials and Methods

Materials

The human osteosarcoma cell line MG-63 was obtained from the American Type Culture Collection (Bethesda, MD). Rabbit antiserum against decorin core protein was obtained as described previously (9). Goat antibody against rabbit IgG conjugated with horseradish peroxidase was from Bio-Rad (Munich, Germany). The following materials were purchased from the suppliers indicated: [*N*-sulfonate- 35 S]heparin (8,000 M_r) and sodium [35 S]sulfate (carrier free) (Amersham-Buchler GmbH, Braunschweig, Germany); heparin-Sepharose CL-6B 1% (Pharmacia Fine Chemicals, Freiburg, Germany); nitro-

cellulose membranes (BA 83, 0.2 μm), (Schleicher und Schuell, Dassel, Germany); chondroitin ABC lyase 1% (Seikagaku Kogyo, Tokyo, Japan); heparin from hog intestinal mucosa and dextran sulfate 500 1% (Serva, Heidelberg, Germany); chondroitin-4-sulfate and chondroitin-6-sulfate, sodium deoxycholate, pepstatin A, leupeptin, and trifluoromethane sulfonic acid 1% (Sigma Chemical Co., Deisenhofen, Germany).

Decorin was prepared from the secretions of human skin fibroblasts by anion-exchange chromatography on a Bio-Gel TSK DEAE-5 PW HPLC column as described previously (12). [^{35}S]Sulfate-labeled decorin was prepared analogously after incubation of fibroblasts in the presence of 0.37 MBq/ml of [^{35}S]sulfate for 72 h. In these incubations streptomycin sesquisulfate was omitted, and FCS, dialyzed against 0.15 M NaCl, was added to a final concentration of 4% (vol/vol).

Decorin-derived glycosaminoglycan chains were obtained after a β -elimination reaction. The proteoglycan was adjusted to 0.15 M NaOH/1 M NaBH₄ and incubated for 18 h at 37°C. The reaction was stopped by adding 1 M acetic acid to pH 5. The solution was made 10% (vol/vol) with FCS and dialyzed against endocytosis medium (see below).

Dermatan sulfate fractions differing in their iduronic acid content were obtained from bovine skin as described (7). Crude glycosaminoglycans were dissolved in 0.25 M acetic acid containing 0.14 M calcium acetate and subjected to differential ethanol precipitation. The iduronic acid-rich fraction precipitating at an ethanol concentration of 18% (vol/vol) was subjected to a β -elimination reaction as described above. After neutralization, the reaction mixture was dialyzed against 20 mM Tris/HCl, pH 7.4, containing 0.15 M NaCl and chromatographed on a DEAE-Trisacryl column (Serva, Heidelberg, Germany) as described previously (9). The glycosaminoglycan fraction was dialyzed against water and lyophilized. Heparan sulfate from bovine aorta was obtained as described previously (27).

Preparation of Decorin/Heparin-binding Proteins

Subcellular fractions enriched in endosomes were prepared from osteosarcoma cells as described previously (12). For Western blotting experiments, the sucrose gradient step was occasionally omitted. In these cases, the vesicular fraction of the post-mitochondrial supernatant was spun down by centrifugation for 1 h at 105,000 *g* at 4°C and then dissolved in sample buffer (19).

Further purification of binding proteins was achieved on a heparin-Sepharose CL-6B column. Approximately 500 μg of endosomal proteins were dissolved in 0.1 M Tris/HCl buffer, pH 7.4, 0.5% (wt/vol) Zwittergent 3-12, 10.1 μM leupeptin, and 7.3 μM pepstatin A (buffer A) containing 0.15 M NaCl and applied to a 4-ml column of the affinity matrix which had been equilibrated with the same buffer. After washing the column with 20 ml of this buffer, a linear gradient of 40 ml of 0.15–1 M NaCl in buffer A was applied. Fractions of 1.15 ml were collected, concentrated to a volume of about 100 μl in a Speed Vac concentrator (Bachofen, Reutlingen, Germany), and proteins were precipitated at 4°C by addition of 200 μl of 17% (wt/vol) TCA. The precipitated material was washed with 500 μl of methanol and dissolved in sample buffer as above.

Binding Experiments

Decorin and heparin binding was studied after SDS-PAGE (19, as modified in 11) followed by Western blotting onto nitrocellulose membranes (37). After blotting, the membranes were rinsed with 20 mM Tris/HCl buffer, pH 7.4, containing 0.15 M NaCl (buffer B) and incubated overnight at 4°C with 3% (wt/vol) BSA in this buffer (blocking solution). The buffer was then replaced by fresh buffer containing additionally $1\text{--}5 \times 10^5$ cpm/ml of either [^{35}S]sulfate-labeled decorin or heparin. Binding was allowed to occur over a period of 60–90 min at ambient temperature. The membranes were then washed seven times for 5 min each with buffer B, dried, and exposed for autoradiography to a preflashed Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY) at -80°C .

Immunochemical evidence for binding was obtained as follows. After Western blotting and treatment with blocking solution, nitrocellulose membranes were incubated with decorin, decorin core proteins, and deglycosylated core protein, respectively, at a concentration of ~ 0.3 nmoles/ml in blocking solution. The membrane was washed with buffer B and further processed by successive incubations with rabbit-antidecorin core protein antiserum, diluted 1:500 in blocking solution, and goat-antirabbit IgG antibody conjugated with HRP, diluted 1:1,000 in blocking solution. Staining was in buffer B/methanol (5:1, vol/vol), containing 2.8 mM 4-chloro-1-naphthol and 4.9 mM H₂O₂.

Endocytosis

Endocytosis by cultured human skin fibroblasts of decorin, dermatan sulfate, and heparin, respectively, was determined exactly as described (8). A pH of 7.2 was maintained by lowering the NaHCO₃ concentration of the culture medium to 1.6 g/l and by addition of 10 mM *N*-(2-hydroxyethyl) piperazine-*N'*-2-ethanesulfonic acid. Since endocytosis of proteoglycans and glycosaminoglycan chains is followed by intralysosomal degradation and concomitant release of inorganic sulfate into the culture medium, endocytosis of [^{35}S]sulfate-labeled macromolecules is represented by the sum of intracellular radioactivity and ethanol-soluble radioactivity in the culture medium. Degradation is defined as the sum of the intra- and extracellular amount of ethanol-soluble radioactivity over the total amount of endocytosed material.

To follow the recycling of internalized heparin, cells were washed twice with calcium-free HBSS and once with PBS, both prewarmed to 37°C. Cells were then incubated for 90 min at 37°C with [*N*-sulfonate- ^{35}S]heparin in PBS containing additionally 1% (wt/vol) BSA. After washing the cells three times with PBS precooled to 4°C, surface-bound heparin was desorbed in the cold by three washes, for 20 min each, with PBS containing 100 $\mu\text{g}/\text{ml}$ of nonradioactive heparin. Finally, cells were returned to 37°C by a short wash with prewarmed PBS, and then incubated for 30 min at 37°C with PBS containing 100 $\mu\text{g}/\text{ml}$ of unlabeled heparin. In control incubations, the last steps were performed at 4°C. Intracellular radioactivity was then obtained by dissolving the cell layer in 1 M NaOH and neutralization with 1 ml of 1 M acetic acid.

Other Methods

Enzymatic digestion of decorin with chondroitin ABC lyase (30) was performed exactly as described. Deglycosylation of decorin core protein with trifluoromethane sulfonic acid was performed according to Sojar and Bahl (36), 33.3% (vol/vol) of anisol was included in the reaction mixture. Hexuronic acids (1) and protein (2) were quantitated as stated previously. Silver staining of SDS-polyacrylamide gels was performed according to Merrill et al. (26).

Results

Binding of Heparin to Decorin-receptor Proteins

Previous work has shown that intact decorin binds to endosomal proteins of 51 and 26 kD and that glycosaminoglycan-free core protein is able to inhibit this interaction (12). It has, therefore, been postulated that the interaction of decorin with the 51- and 26-kD proteins is mediated via the protein moiety of the proteoglycan. However, the binding of the isolated core protein had not yet been demonstrated directly. Since it will be shown below that highly negatively charged glycosaminoglycans bind to these receptor proteins, too, it seemed necessary to verify the proposal of a core protein-receptor interaction by a more direct method. With the aid of a monospecific antiserum against decorin core protein, binding of intact decorin, of glycosaminoglycan-free core protein and of completely deglycosylated core protein could indeed be demonstrated qualitatively on a Western blot of endosomal proteins from osteosarcoma cells (Fig. 1). From the results of several experiments it appeared that the intact and the chondroitin ABC lyase-degraded proteoglycan exhibited similar binding properties. However, treatment with trifluoromethanesulfonic acid, which led to the appearance of the carbohydrate-free core protein of 36 kD (result not shown), had a negative influence on the binding properties. There was only weak binding to the 26-kD protein, but additional binding to a 28-kD protein. This observation has not yet been studied further. It seems possible that oligosaccharides are involved in binding to the 26-kD protein. In previous studies, however, no indication was obtained for the involve-

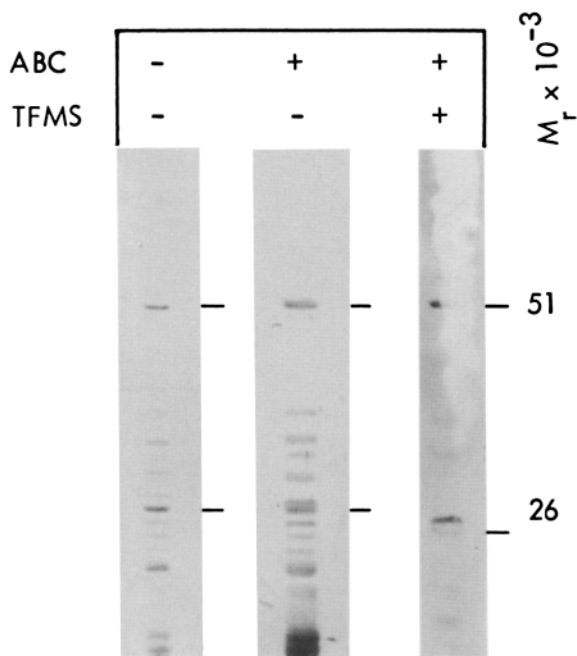


Figure 1. Indirect immunostaining of decorin-binding proteins. Intact decorin, decorin core protein, and deglycosylated core protein, respectively, were allowed to bind to blotted endosomal proteins. Binding was demonstrated by immunostaining with an anti-decorin core protein antiserum and anti-IgG conjugated to HRP. *ABC*, treatment with chondroitin ABC lyase; *TFMS*, treatment with trifluoromethane-sulfonic acid.

ment of oligosaccharides in the endocytotic process (8). Several other proteins with decorin-binding properties that are seen in Fig. 1 are probably derived by proteolysis during preparation of the endosomes (12).

Surprisingly, binding of decorin to the 51- and 26-kD-proteins could be strongly inhibited by several glycosaminoglycans (Fig. 2). At a dose of 10 $\mu\text{g/ml}$, heparin and dextran sulfate abolished binding almost completely. Dermatan sulfate was somewhat less effective, but chondroitin-4-sulfate occasionally even enhanced decorin binding. The undersulfated heparan sulfate (0.7 $\mu\text{mol sulfate}/\mu\text{mol hexuronic acid}$) was slightly inhibitory.

Direct binding of [^{35}S]sulfate-labeled heparin to endosomal proteins demonstrated an interaction of this polyanion with the 51- and 26-kD proteins and with a variety of other proteins (Fig. 3). There was no effective competition for heparin binding by several other glycosaminoglycans including dermatan sulfate and heparan sulfate at the doses used. When the 51-kD protein on a Western blot of endosomal proteins was first incubated with [^{35}S]sulfate-labeled decorin, unlabeled heparin (5 $\mu\text{g/ml}$) released 38% of bound radioactivity within 5 min, and 77% within 15 min. In a different experimental approach, solubilized endosomal proteins were chromatographed on a heparin-Sepharose CL-6B column (Fig. 4). Adsorbed proteins were eluted by applying a linear gradient from 0.15–1.0 M NaCl. The 26-kD protein was desorbed at a NaCl concentration of about 0.5 M and the 51-kD protein at a NaCl concentration of about 0.7 M. Decorin-binding proteins eluting at a lower salt concentration were not found.

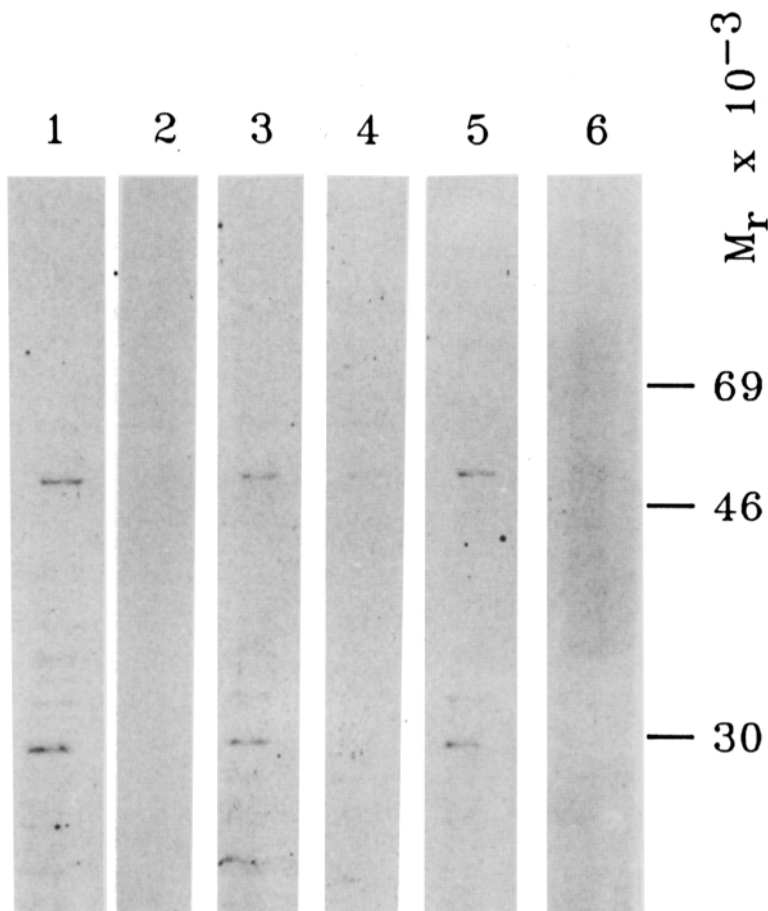


Figure 2. Effect of different glycosaminoglycans on decorin binding to endosomal proteins. 20 μg of endosomal proteins from human MG-63 osteosarcoma cells were subjected to SDS-PAGE under nonreducing conditions and Western blotting. Binding of [^{35}S]sulfate-labeled decorin was performed either in the absence (lane 1) or presence of 10 $\mu\text{g/ml}$ of the following glycosaminoglycans: heparin (lane 2), heparan sulfate (lane 3), β -eliminated dermatan sulfate chains (lane 4), chondroitin-4-sulfate (lane 5), and dextran sulfate (lane 6).

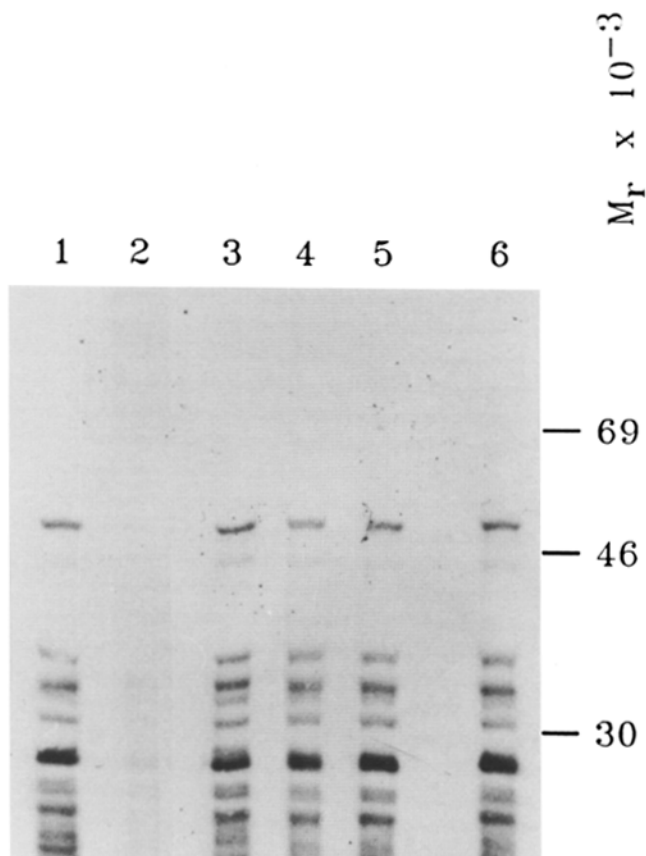


Figure 3. Binding of heparin to endosomal proteins. 30 μg of endosomal proteins were blotted onto nitrocellulose as described in the legend of Fig. 1 and incubated with [N -sulfonate- ^{35}S]heparin either in the absence (lane 1) or presence of 10 $\mu\text{g}/\text{ml}$ of the following glycosaminoglycans: heparin (lane 2), heparan sulfate (lane 3), β -eliminated dermatan sulfate chains (lane 4), chondroitin-6-sulfate (lane 5), and chondroitin-4-sulfate (lane 6).

Inhibition of Decorin Endocytosis by Heparin

The binding of heparin to decorin receptor proteins suggested an inhibitory effect of heparin on the uptake of the proteoglycan by intact cells. Indeed, endocytosis of [^{35}S]sulfate-labeled decorin could be inhibited in part by low concentrations of heparin (Fig. 5). A minor inhibitory effect was found for iduronic acid-rich dermatan sulfate, too. Exposing the cells for 6 h to labeled decorin (750,000 cpm/ml), heparin (10 $\mu\text{g}/\text{ml}$) led to an inhibition of decorin uptake by 48%, and iduronic acid-rich dermatan sulfate chains at the same concentration by 12%. In the presence of chondroitin-4-sulfate, decorin endocytosis remained unaltered.

Endocytosis of Heparin

The high-affinity binding of [^{35}S]sulfate-labeled heparin to endosomal proteins let us assume a high rate of endocytosis of this polysaccharide by fibroblasts. A comparative study of the uptake of heparin and decorin, however, indicated that decorin is endocytosed at least 30 times more efficiently than heparin (Fig. 6). Endocytosis of decorin is followed by transport to lysosomes and intralysosomal degradation. In the ex-

periment described in Fig. 6, 75–79% of the endocytosed proteoglycan had been degraded as judged by the appearance of inorganic [^{35}S]sulfate. On the contrary, only 8–10% of endocytosed heparin had been depolymerized.

Dermatan sulfate chains are even less efficiently endocytosed than heparin. When cells were allowed to endocytose either decorin or dermatan sulfate chains derived from the same decorin preparation by β elimination, the clearance rates were 95.2 $\mu\text{l}/\text{h}$ and mg cell protein for decorin and only 0.8 $\mu\text{l}/\text{h}$ and mg for dermatan sulfate.

pH Insensitivity of Heparin Binding to Endosomal Proteins

In an analogous experiment as that described in Fig. 3, endosomal proteins were exposed to [^{35}S]sulfate-labeled heparin at pH 7.4. Subsequent incubation at pH 4.0 did not result in a measurable release of bound ligand (Fig. 7). This is in sharp contrast to the behavior of bound decorin which is almost completely released at acid pH (12).

Replacement of Internalized Heparin

The results described in the previous sections suggest that heparin could be bound by a high-affinity endocytosis receptor, but is not released from the receptor at the pH being present in the compartment of uncoupling of receptor and ligand (CURL). It seemed, therefore, possible that internalization of heparin is followed by its reappearance at the plasma membrane. To test this hypothesis, fibroblasts were exposed to [^{35}S]sulfate-labeled heparin at 37°C. The cells were then treated at 4°C with an excess of unlabeled heparin to exchange labeled ligand at the plasma membrane. Further incubation was again at 37°C in the presence of unlabeled heparin. It is shown in Fig. 8 that, depending on the dose of originally applied heparin, 40–90% of internalized heparin reappeared at the plasma membrane and could be desorbed by unlabeled heparin.

Discussion

The results described in this communication provide direct evidence that the core protein of the small proteoglycan decorin is interacting with the 51- and 26-kD proteins which are involved in receptor-mediated endocytosis of decorin. The pI value of 9.8 for the unsubstituted decorin core protein is remarkably high (18). It was, therefore, unexpected that the polyanion heparin bound to the same proteins. Though the presence of an acidic domain found in the tertiary structure of decorin core protein, which binds to the same site as heparin, could not be excluded, it appears more likely that heparin and decorin core protein are bound by adjacent sites on the receptor proteins. Adjacent binding sites for decorin core protein and heparin have been proposed to exist on the NH_2 - and COOH -terminal heparin-binding domains of fibronectin (32).

Competition experiments suggested that other glycosaminoglycans than heparin could interact with the 51- and 26-kD proteins. Undersulfated heparan sulfate was only slightly effective, but heparan sulfate from fibroblasts contains heparin-like domains (41) which should bind more strongly to these proteins. Dermatan sulfate was an effective inhibitor of decorin binding. Nevertheless, the glycosaminoglycan moiety

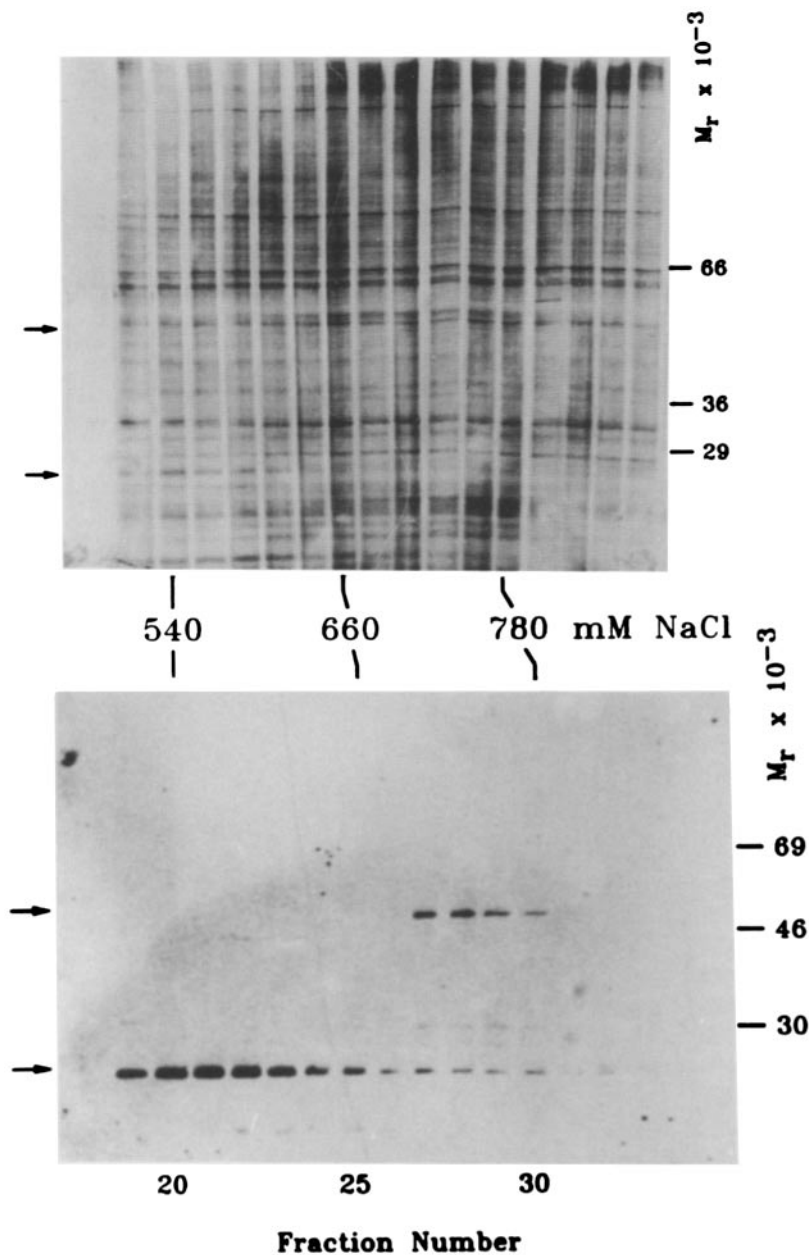


Figure 4. Heparin-affinity-chromatography of endosomal proteins. Endosomal proteins from MG-63 osteosarcoma cells were subjected to chromatography on a heparin-Sepharose CL-6B column. Proteins eluted from the column at the indicated NaCl concentrations were separated by SDS-PAGE and either stained with AgNO_3 or, after blotting onto nitrocellulose, incubated with $[^{35}\text{S}]$ sulfate-labeled decorin. Arrows indicate 51- and 26-kD proteins, respectively.

of decorin should play only a minor functional role in mediating the endocytosis of the proteoglycan. Free dermatan sulfate chains were only slightly inhibitory for decorin uptake, and their clearance rate amounted to as little as 1% of that of decorin (this paper, and 27).

It had been shown previously that heparan sulfate can be internalized by fibroblasts in a dose-dependent and saturable manner, albeit much less efficiently than decorin (17). To our knowledge, studies on the endocytosis of heparin by fibroblasts have not been published in the literature. Vascular smooth muscle cells were able to internalize a minor proportion of surface-bound heparin. After initial rapid uptake, the rate slowed down substantially (3). Clearance and degradation rates, however, had not been given. Our data suggest that the low efficiency of heparin uptake does not result from its low affinity to receptor proteins but from the stability of

the receptor ligand complex at acid pH. Thus, heparin appears to be transported along the recycling endocytic pathway in intact cells similarly to the transport of transferrin (5, 16). Consequently, after internalization of heparin the polysaccharide reappears on the cell surface and can be desorbed by an excess of exogenously added heparin. Decorin, on the contrary, binds to the same receptor proteins but is transported along the lysosomal endocytic pathway and becomes degraded to its monomeric constituents.

It should be noted that a small proportion of heparin is also transferred to lysosomes. From the results of the experiment designed to demonstrate heparin recycling, it appears likely that heparin-binding proteins of high affinity are responsible for the recycling pathway and those of lower affinity for the endocytic pathway.

The major unresolved question raised by the present data

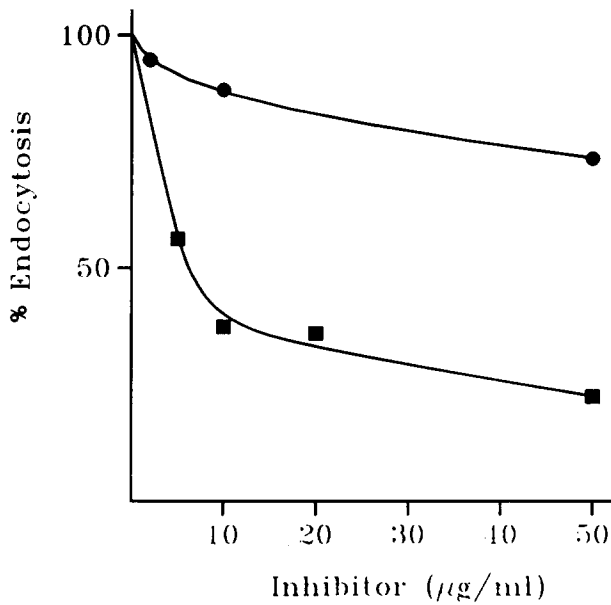


Figure 5. Inhibition of the endocytosis of decorin by heparin and dermatan sulfate. Human skin fibroblasts were incubated for 6 h with [³⁵S]sulfate-labeled decorin (750,000 cpm/ml for inhibition by dermatan sulfate, 100,000 cpm/ml for inhibition by heparin) in the presence of the indicated concentrations of β -eliminated dermatan sulfate chains (●) and heparin (■), respectively.

concerns the biological relevance of the competition of decorin and heparin for binding to the same receptor proteins. Heparin is certainly only a minor component in the extracellular matrix, and there is a wealth of heparin-binding proteins (see 22, 23, and 29 for reviews) which include several extracellular matrix proteins and growth factors in addition to plasma-derived proteins. This multitude of proteins should compete for heparin binding with the receptor proteins. As stated above, heparin-like heparan sulfate fractions

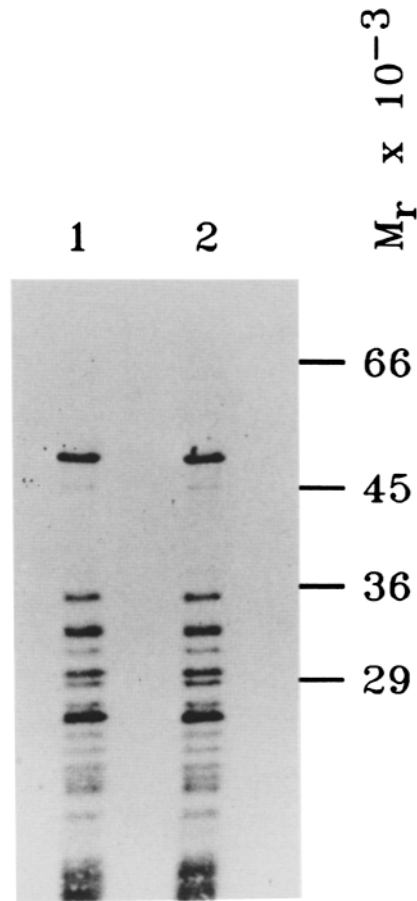


Figure 7. pH sensitivity of heparin binding to endosomal proteins. Western blots of endosomal proteins were incubated with [*N*-sulfonate-³⁵S]heparin at pH 7.4 and washed five times for 5 min each with 20 mM Tris/HCl, pH 7.4, containing 0.15 M NaCl (lane 1), or with 50 mM sodium acetate, pH 4.0, containing 100 mM NaCl (lane 2).

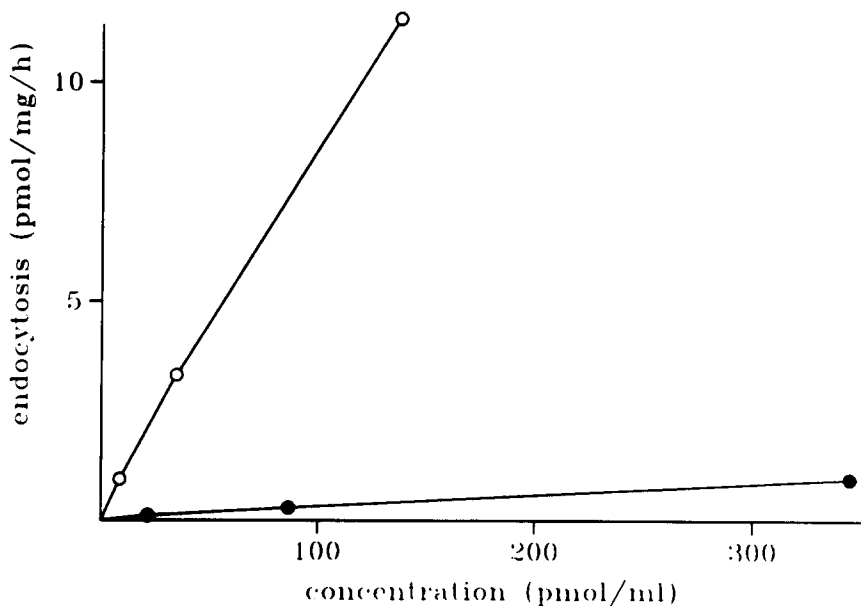


Figure 6. Endocytosis of decorin and heparin by human skin fibroblasts. Cells were incubated for 24 h with [³⁵S]sulfate-labeled decorin ($\sim 5.8 \times 10^6$ cpm/nmole) and [*N*-sulfonate-³⁵S]heparin ($\sim 2.3 \times 10^6$ cpm/nmole), respectively. (●) heparin; (○) decorin.

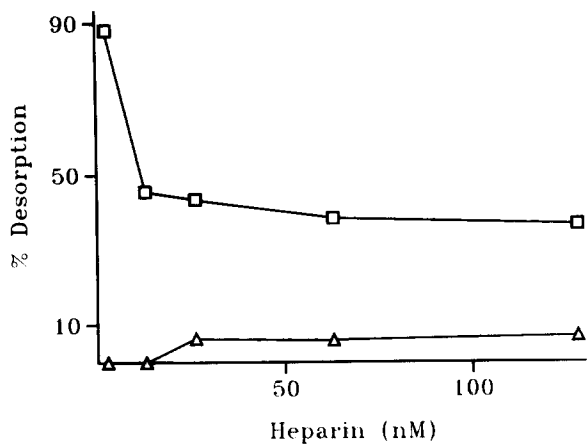


Figure 8. Recycling of endocytosed heparin. Cells were allowed to bind and internalize [*N*-sulfonate-³⁵S]heparin at 37°C for 90 min. The cells were cooled to 4°C, and surface-bound ligand was desorbed by an excess of unlabeled heparin. Cells were then warmed to 37°C and incubated for 30 min at this temperature in the presence of unlabeled heparin, competing for labeled heparin reappearing at the cell surface (□). Control cells were kept at 4°C during this final desorption step (Δ). The portion of removable heparin over the total amount of heparin is shown.

could be expected also to serve as competitors for binding. The existence of membrane-intercalated and -associated heparan sulfate proteoglycans (15, 25) could facilitate the complex formation with the receptor. Since a heparan sulfate core protein carries several glycosaminoglycan chains and participates in the transmembrane linking of intracellular cytoskeletal components to extracellular matrix constituents (20) it could possibly retard the movement of the endocytosis receptor into intracellular compartments. In preliminary experiments we observed indeed that enzymatic removal of heparan sulfate from the cell surface facilitated subsequent uptake of decorin. Membrane-associated heparan sulfate could, therefore, be involved in the regulation of decorin turnover. On the other hand, the presence of decorin-binding proteins in the extracellular matrix can also interfere with decorin uptake (10, 31). Further studies are needed to elucidate the complex interactions between decorin and heparin/heparan sulfate and the endocytosis receptor.

We thank D. Holtfreich and M. Kowalak for skillful technical assistance.

This work was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 310, Teilprojekt B2).

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