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Binding of transferrin to the core protein of fibroblast proteoheparan sulfate

(cell surface/heparan-sulfate lyase/protein A/proteoglycan/transferrin receptor)

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ABSTRACT Cell-surface-associated proteoheparan sulfate from confluent human skin fibroblasts appears to consist of two disulfide-bonded polypeptides of M_r ≈90,000. The transferrin receptor, a ubiquitous cell-surface component of proliferating cells, also consists of two subunits of M_r 90,000 linked by S—S bonds. Radiolabeled proteoheparan sulfate mixed with holotransferrin or apotransferrin at pH 4–5 followed by rabbit anti-human transferrin was adsorbed onto protein A-Sepharose to ≈80–90%. At pH 7.5 apotransferrin bound ≈40% of the proteoglycan, whereas ≈80% was bound to holotransferrin. Trypsin digestion of the proteoglycan markedly lowered its ability to bind transferrin. However, binding was essentially unaffected by heparan-sulfate lyase treatment and after reduction and alkylation. Over 90% of the ^{3}H activity of an L-[^{3}H]leucine-labeled proteoglycan was recovered by immunoprecipitation (transferrin·antitransferrin) of a heparan-sulfate lyase digest of the proteoglycan. The immunoprecipitated core protein had an apparent M_r of 150,000 before reduction and M_r of 90,000 after reduction of disulfide bonds. The core protein of the proteoglycan was recognized by the monoclonal antibody B3/25, which is known to be receptor specific. The present findings suggest that the core polypeptides of proteoheparan sulfate and the transferrin receptor may be identical or closely similar.

Cell surface glycoproteins and proteoglycans of animal cells have been proposed to play a role in cell–cell and cell–matrix interactions, growth regulation, and uptake of nutrients (1). The transferrin receptor, which participates in uptake of iron by growing cells, is a ubiquitous membrane-bound glycoprotein consisting of two glycosylated polypeptides joined with disulfide bonds (M_r ≈90,000 each) (2). Many eukaryotic adherent cells also synthesize a heparan sulfate-substituted glycoprotein (proteoheparan sulfate) that is deposited in the pericellular coat (for references, see ref. 3). We have previously isolated and characterized radiolabeled proteoheparan sulfates from confluent cultures of human embryonic skin fibroblasts (3). The proteoglycan isolated from the cell monolayer was M_r 350,000, whereas that of the culture medium was smaller (M_r 140,000). Reduction and alkylation of the cell-derived macromolecule gave rise to a component with an apparent M_r of 140,000, while the medium-derived form was not affected. Heparan-sulfate lyase treatment of the cell-derived proteoglycan yielded a core protein of apparent M_r 150,000, which, upon reduction, afforded polypeptides of M_r 80,000–100,000 (4). It was suggested that the core protein consists of two subunits linked by disulfide bonds. The structural similarity between the transferrin receptor glycoprotein and the core protein of fibroblast proteoheparan sulfate prompted us to investigate whether or not the latter could bind transferrin.

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MATERIALS AND METHODS

Materials. Human embryonic skin fibroblasts in confluent cultures were incubated with L-[^{3}H]leucine and $^{35}SO_4^{2-}$, D[^{3}H]glucosamine and $^{35}SO_4^{2-}$, or only $^{35}SO_4^{2-}$ for 72 hr and proteoglycans were isolated from the medium and a 4 M guanidinium chloride extract of the cell layer in the presence of proteinase inhibitors (3). Proteoheparan sulfate was purified by isopycnic density-gradient centrifugation in CsCl/4 M guanidinium chloride followed by gel-permeation and ion-exchange chromatography after degrading contaminating proteodermatan sulfate with chondroitin ABC lyase in the presence of proteinase inhibitors (3). Proteodermatan sulfate (type II) was from bovine sclera (5). Heparan-sulfate lyase (heparitinsulfate lyase; EC 4.2.2.8) from *Flavobacterium heparinum* was a product of Seikagaku Kogyo (Tokyo, Japan). Ovalbumin, ovomucoid, human albumin, transferrin (substantially iron-free), α_1-acid glycoprotein, α_1-antitrypsin, and α_2-macroglobulin were from Sigma. Rabbit immunoglobulin fractions against the human serum proteins and against mouse immunoglobulins were from Dakopatts (Copenhagen), and an antiserum against the proteodermatan sulfate was raised in this laboratory. The following monoclonal antibodies against the transferrin receptor were used: B3/25 (Hybritech, San Diego, CA), OKT9 (Ortho), and L5/1 (gift from T. Kalland, Dept. of Anatomy, Univ. of Lund). Highly purified human lactoferrin and an antiserum against it were a generous gift of A. Franzén of this department. Protein A-Sepharose, Sepharose gels, as well as proteins for calibration were purchased from Pharmacia. Desferrioxamine was from CIBA Pharmaceutical. Other chemicals were obtained from sources listed previously (3).

Methods. To test for transferrin binding, radiolabeled proteoheparan sulfate (2000 dpm), from the cell layer or the culture medium, in 50 μl of 10 mM KPO_4 buffer (pH 7.5) containing 0.15 M NaCl, 0.1% (vol/vol) Triton X-100, and 5 μg of ovalbumin per ml (if not stated otherwise) was mixed with 10 μl of human transferrin (1 mg/ml) in the same buffer (final concentration of transferrin = 0.17 mg/ml). In some experiments the buffer contained 0.5 M NaCl and/or transferrin was replaced with other proteins at the same concentration. After incubation at 37°C for 20 min the solutions were adjusted to pH 5.0 by the addition of 25 μl of 1 M sodium citrate buffer (pH 5.0) and 50 μl of 50 mM KPO_4 buffer (pH 5.0) containing 0.15 or 0.5 M NaCl and 0.1% (vol/vol) Triton X-100 (buffer A). Rabbit immunoglobulins against transferrin or the various serum proteins or antisera against the other proteins were added in sufficient amounts to precipitate all of the protein and the immune reaction was allowed to proceed for 1 hr at 4°C. The solutions were then applied to columns containing ≈0.1 ml of protein A-Sepharose in buffer A. The gel was first eluted with 0.5 ml each of buffer A and then with 50 mM KPO_4 buffer (pH 5.0) containing 0.5 M NaCl and 0.5% (vol/vol) Triton X-100. Bound material was finally displaced with 4 M guanidinium chloride containing 1% (vol/vol) Triton X-100 or with 1% (wt/vol) $NaDodSO_4$.

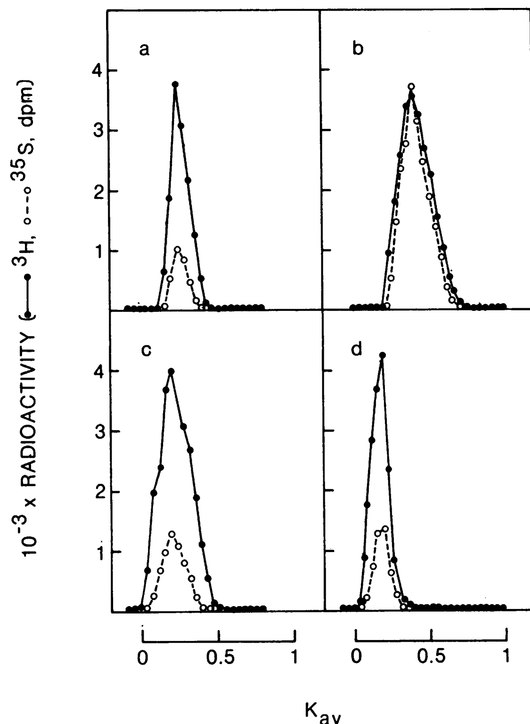

FIG. 1. Gel chromatography of proteoheparan sulfate from the cell monolayer (*a*, *c*, and *d*) and from the medium (*b*). The [^{3}H]leucine- and $^{35}SO_4^{2-}$-labeled proteoglycans were purified, and chromatography was performed at room temperature on a column (0.6 × 150 cm) of Sepharose CL-4B eluted with 50 mM NaOAc buffer (pH 5.8) containing 4 M guanidinium chloride (*a* and *b*), the same solvent containing 1% (vol/vol) Triton X-100 (*c*), or 1% (wt/vol) $NaDodSO_4$ in 50 mM $NaHCO_3$ buffer (pH 7.0) (*d*). In *d*, the sample was heated at 60°C for 40 min in 2% (wt/vol) $NaDodSO_4$ before chromatography. Fractions were assayed for radioactivity: ●, ^{3}H; ○, ^{35}S.

After the addition of 5 ml of Instagel radioactivity was measured by liquid scintillation.

Monoclonal antibodies to the transferrin receptor (B3/25, OKT9, L5/1) were tested for binding to radiolabeled proteoheparan sulfate as follows. [^{3}H]Leucine-labeled proteoglycans or derivatives thereof were incubated with 1 μg of antibody in 50 μl of 50 mM Tris buffer (pH 8.0) for 1 hr at 4°C. After addition of 100 μl of rabbit anti-mouse immunoglobulins the mixtures were passed through protein A-Sepharose, which was eluted as described above.

The heparan sulfate side chains were removed from the core protein of the proteoglycan by digestion with heparan sulfate-lyase (6 international milliunits/ml) in 3 mM $Ca(OAc)_2$ (pH 7.0) containing 0.5 mg of ovomucoid per ml at 37°C for 4 hr in tubes that were pretreated with a solution of ovalbumin (0.5 mg/ml).

Reduction of disulfide bonds was performed in 50 mM NaOAc buffer (pH 5.8) containing 4 M guanidinium chloride and 10 mM dithiothreitol or 50 mM $NaHCO_3$ buffer (pH 7.0) containing 1% (wt/vol) $NaDodSO_4$ and 10 mM dithiothreitol, both at 37°C for 5 hr. Alkylation was subsequently achieved by the addition of a 2.5 M excess of iodoacetamide over dithiothreitol and by keeping the solution in the dark overnight. Trypsin digestion was performed in 200 μl of 8 mM $NaPO_4$ buffer (pH 7.4) containing 137 mM NaCl, 3 mM KCl, and 10 μg of enzyme for 24 hr at 37°C.

RESULTS AND DISCUSSION

The cell-derived radiolabeled proteoheparan sulfate chromatographed on Sepharose CL-4B with a K_{av} of 0.25 in 4 M guanidinium chloride (Fig. 1*a*). The addition of 1% (vol/vol) Triton X-100 to the latter solvent did not change the elution position (Fig. 1*c*) and after heating in the presence of 2% (wt/vol) $NaDodSO_4$ followed by chromatography in 1% (wt/vol) $NaDodSO_4$ the K_{av} was 0.2 (Fig. 1*d*). No ^{3}H emerged with the retarded fractions, suggesting the absence of nonproteoglycan proteins. As shown elsewhere (3, 4) the

Table 1. Binding of [^{3}H]leucine-labeled proteoheparan sulfates to transferrin and other proteins

Exp.	Proteoheparan sulfate from	Addition: Protein	Addition: Antibody against	Binding to protein A, % of added ^{3}H*	
1	Cell	None	None	5	
2	Cell	Transferrin	None	1	
3	Cell	None	Transferrin	18	
4	Cell	Transferrin	Transferrin	80 ± 6†	(82)
5	Cell	Transferrin	Albumin	11	
6	Cell	Transferrin	Calf serum	7	
7	Cell	α_1-Acid glycoprotein	α_1-Acid glycoprotein	35	(5)
8	Cell	α_1-Antitrypsin	α_1-Antitrypsin	20	(6)
9	Cell	α_2-Macroglobulin	α_2-Macroglobulin	16	(3)
10	Cell	Albumin	Albumin	28	(8)
11	Cell	Lactoferrin	Lactoferrin	6	
12	Cell	Proteodermatan sulfate	Proteodermatan sulfate	1	
13	Cell	None	Proteodermatan sulfate	2	
14	Medium	Transferrin	Transferrin	10	(9)
15	Medium	Albumin	Albumin	25	(5)
16	Medium	α_1-Acid glycoprotein	α_1-Acid glycoprotein	34	(4)
17	Medium	α_1-Antitrypsin	α_1-Antitrypsin	25	(8)
18	Medium	α_2-Macroglobulin	α_2-Macroglobulin	24	

Binding of transferrin has been tested down to 10 ng of transferrin per ml of reaction mixture. The result was the same as in experiment 4.

*Values within parentheses were obtained when binding was assayed in the presence of 0.5 M NaCl.

†Mean ± SEM.

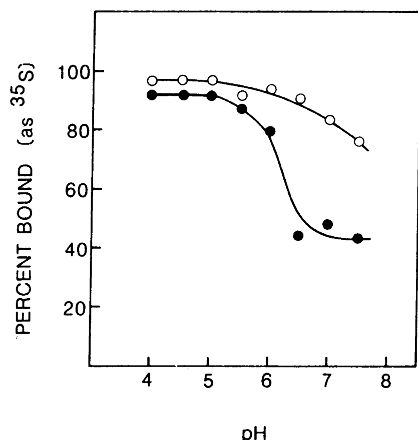

FIG. 2. Binding of ^{35}S-labeled proteoheparan sulfate to transferrin at various pH values in the absence (○) or presence (●) of desferrioxamine. The assay was performed as described in the text, except that the pH was maintained at the initial value. Buffers at pH 4, 4.5, 5, and 5.5 were composed of 50 mM NaOAc, 0.15 M NaCl, and 0.1% (vol/vol) Triton X-100; buffers at pH 6, 6.5, 7, and 7.5 contained 50 mM $NaPO_4$ instead of NaOAc. The concentration of desferrioxamine was 50 μM.

K_{av} values observed correspond to molecular weights on the order of M_rs 300,000–400,000. The medium form of proteoheparan sulfate had a K_{av} of 0.4 (Fig. 1b) corresponding to $M_r \approx 150,000$. Both proteoglycans were bound quantitatively (measured as ^3H as well as ^{35}S radioactivity) to DEAE-cellulose in the presence of 0.2 M NaOAc buffer (pH 5.8) containing 6 M urea and 10 mM dithiothreitol (results not shown), again suggesting the absence of nonproteoglycan proteins.

Binding of Transferrin to Proteoheparan Sulfate. The proteoheparan sulfate from the culture medium as well as that of the cell layer were tested for transferrin binding by immunoprecipitation of the transferrin component of a putative transferrin·proteoglycan complex (6). As shown in Table 1 the cell-derived proteoglycan was bound to ≈80% to protein A in the presence of transferrin and antitransferrin (experiment 4). When either transferrin or antitransferrin was omitted or if unrelated antisera were used, 1–18% of the labeled material was bound (experiments 1–3, 5, and 6), suggesting that transferrin can bind to the proteoheparan sulfate derived from the cell layer. The specificity was tested by mixing the proteoglycan with other proteins and their corresponding antibodies (experiments 7–11). The extent of binding to various plasma proteins ranged from 6% to 35% in the presence of 0.15 M NaCl and from 3% to 8% in the presence of 0.5 M NaCl, which we ascribe to ionic interaction between the heparan sulfate side chains and the plasma proteins (7). Lactoferrin, which is structurally related to transferrin, was not bound significantly (experiment 11). Proteoheparan sulfate did not coprecipitate with an immune complex formed between proteodermatan sulfate (8) and an antiserum against the latter (experiments 12 and 13). The medium-derived proteoheparan sulfate showed a low extent of binding to trans-

Table 2. Effect of various treatments on transferrin binding to [^{3}H]leucine-labeled proteoheparan sulfate from the cell layer

Treatment	Binding to protein A, % of added ^{3}H
Heparan-sulfate lyase	82 ± 10*
Reduction and alkylation	75 ± 6*
Trypsin	13

After treatment, all samples were dialyzed against the buffer used in the binding studies.
*Mean ± SEM.

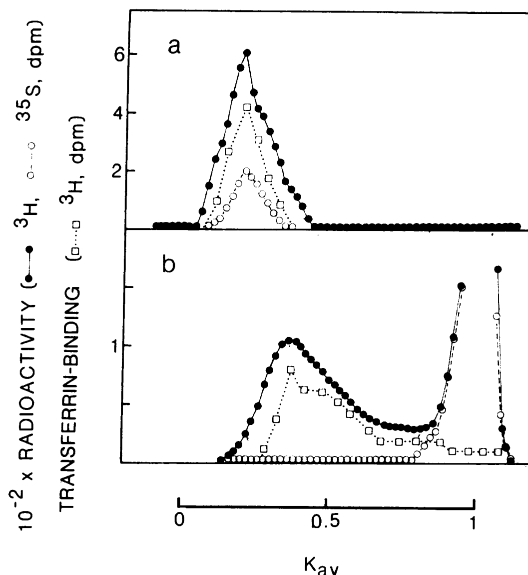

FIG. 3. Cochromatography of transferrin-binding ability and proteoheparan sulfate (a) or its core protein (b). [^{3}H]Glucosamine- and $^{35}SO_4^{2-}$-labeled proteoglycan was chromatographed (in a) on a column (1.8 × 145 cm) of Sepharose CL-4B that was eluted with 50 mM NaOAc buffer (pH 5.8) containing 4 M guanidinium chloride, 1% (vol/vol) Triton X-100, and 5 μg of ovalbumin per ml at a rate of 6 ml/hr. Fractions (≈3.4 ml) were collected by drop-counting and were analyzed for radioactivity and transferrin binding after dialysis against 10 mM KPO_4 buffer (pH 7.5) containing 0.15 M NaCl (see Table 1). The transferrin-binding ability (□) is expressed as the amount of ^{3}H activity in each fraction that was bound to protein A in the presence of transferrin and antitransferrin. In b, the proteoheparan sulfate was chromatographed on the same column after treatment with heparan-sulfate lyase. ●, ^{3}H; ○, ^{35}S.

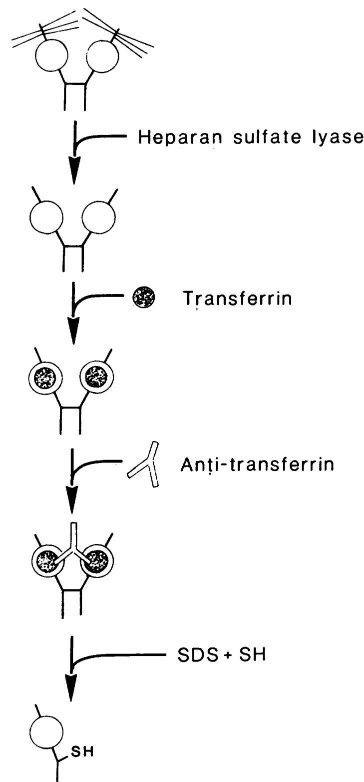

FIG. 4. Isolation of core polypeptides from proteoheparan sulfate by virtue of their transferrin-binding ability. + SH, reducing conditions; SDS, $NaDodSO_4$.

ferrin (experiment 14). The extent of binding of the other serum proteins to the medium form of the proteoglycan was between 24% and 34% in the presence of 0.15 M NaCl and from 4% to 18% in the presence of 0.5 M NaCl (experiments 15–18)—i.e., in the same order as that observed for the cell-derived proteoglycan.

The binding of transferrin to its receptor is dependent both on pH and on the presence of ferric ion in the transferrin molecule (9, 10). Apotransferrin and diferric transferrin (holotransferrin) bind to the receptor with similar affinities at a pH below 5.5, whereas apotransferrin, in contrast to holotransferrin, binds less avidly to its receptor at neutral pH. Since trace amounts of iron, present in all salt solutions, will generate holotransferrin, studies on the binding of apotransferrin to a putative receptor require the addition of desferrioxamine to sequester the iron (9). Therefore, we have also examined the extent of binding of cell-derived proteoheparan sulfate to transferrin at various pH values and in the absence or presence of desferrioxamine (Fig. 2). In the absence of the iron chelator, 78–98% of the proteoglycan was bound over the pH range 4–7.5. However, at pH 6.5–7.5, the extent of binding to apotransferrin was decreased to ≈45%.

Transferrin Is Bound to the Core Protein of Proteoheparan Sulfate. To show that transferrin was bound to the core protein of fibroblast proteoheparan sulfate, the side chains were removed by digestion with heparan-sulfate lyase. This treatment had no effect on the binding of transferrin (Table 2) nor did reduction and alkylation in the presence of guanidinium chloride substantially affect binding, which suggests that both polypeptides of the core protein are able to bind to transferrin. Trypsin digestion extensively erodes the nonglycosylated portions of the core protein of proteoheparan sulfate (3) and yields fragments with clustered heparan sulfate side chains similar in size to the intact medium form of the proteoglycan (3). Both the trypsin-treated, cell-derived proteoheparan sulfate (Table 2) and the medium-derived proteoheparan sulfate (Table 1; experiment 14) showed a low extent of binding to transferrin.

To further corroborate that the binding site for transferrin resides in the core protein of cell-derived proteoheparan sulfate, the gel filtration experiments shown in Figs. 3–5 were performed. The untreated proteoheparan sulfate had a K_{av} of 0.2 (Fig. 3*a*), whereas the [^{3}H]glucosamine-labeled core protein had a K_{av} of 0.3–0.5 (Fig. 3*b*). The transferrin-binding ^{3}H activity was associated with the core protein and not with the ^{3}H- and ^{35}S-labeled heparan sulfate oligosaccharides (K_{av} = 0.9–1 in Fig. 3*b*). In another experiment, [^{3}H]leucine- and $^{35}SO_4^{2-}$-labeled proteoheparan sulfate (see Fig. 4) was first treated with heparan-sulfate lyase and then with transferrin and antitransferrin. Immune complexes adsorbed onto protein A were finally displaced with 1% (wt/vol) $NaDodSO_4$. Whereas all of the radiosulfate emerged with the washings, 94% of the [^{3}H]leucine was recovered in the 1% (wt/vol) $NaDodSO_4$ eluant. The elution profile of this material on Sepharose CL-4B was then compared with that of a heparan-sulfate lyase digest of the proteoglycan. As seen in Fig. 5*b*, the digest that had not been immunoprecipitated yielded a ^{3}H-labeled core protein at K_{av} ≈0.4. The corresponding immu-

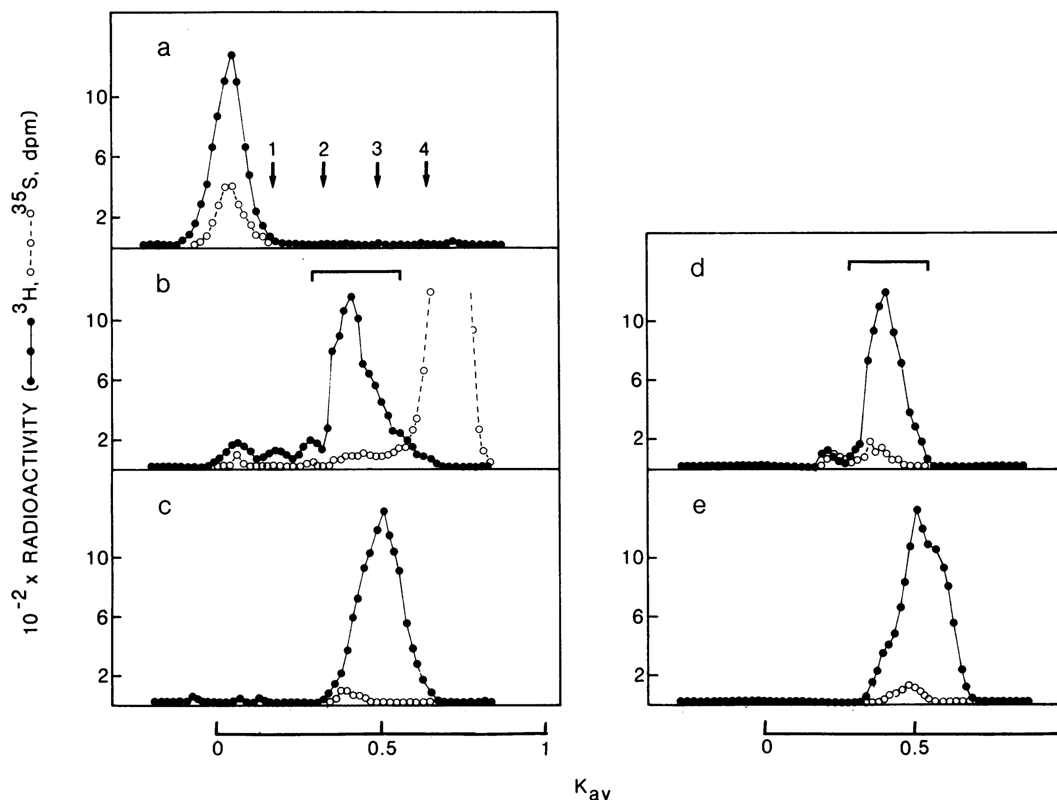

FIG. 5. Gel chromatography of intact proteoheparan sulfate (*a*), the core protein obtained after treatment with heparan-sulfate lyase (*b* and *d*), and the core protein obtained after reduction and alkylation (*c* and *e*). The [^{3}H]leucine- and $^{35}SO_4^{2-}$-labeled proteoglycan was treated with heparan-sulfate lyase. In one case (*b*) the digests were chromatographed directly, and in the other (*d*) the digests were treated with transferrin and antitransferrin and passed over protein A-Sepharose. The core protein was recovered by elution with 1% (wt/vol) $NaDodSO_4$ and chromatographed. Both preparations (*b* and *d*) were recovered (see bar) and subjected to reduction and alkylation in the presence of $NaDodSO_4$ (*c* and *e*). The column (0.6 × 150 cm) contained Sepharose CL-4B and was eluted with 50 mM $NaHCO_3$ buffer (pH 7.0) containing 1% (wt/vol) $NaDodSO_4$. The standards were thyroglobulin, M_r 330,000 (1); ferritin subunit, M_r 220,000 (2); phosphorylase b, M_r 94,000 (3); catalase subunit, M_r 60,000 (4). The K_{av} of each standard was plotted against log molecular weight and a straight line was obtained. The K_{av} for the unreduced core protein corresponded to M_r ≈150,000, whereas the reduced and alkylated core protein was M_r ≈90,000. ●, ^{3}H; ○, ^{35}S.

noprecipitated core protein was eluted in the same position (Fig. 5*d*). Both of these core protein preparations were susceptible to reduction in the presence of $NaDodSO_4$. The single polypeptides had an estimated M_r of 90,000 (Fig. 5 *c* and *e*). The broadness of the peaks may be ascribed to the presence of a variable number of oligosaccharide residues entailing the linkage region between heparan sulfate and the core protein. These oligosaccharides should contain few, if any, sulfate groups.

Similarities Between the Proteoheparan Sulfate Core Protein and the Transferrin Receptor. The relationship between the core protein of proteoheparan sulfate and the transferrin receptor was also investigated by using various monoclonal antibodies (B3/25, OKT9, L5/1) that are known to specifically recognize the receptor glycoprotein (2, 11–13). As shown in Table 3, the intact cell-derived proteoglycan did not react with monoclonal antibody OKT9, L5/1, or B3/25, whereas the B3/25 monoclonal antibody recognized the core protein after removal of the side chains by heparan-sulfate lyase. The OKT9 monoclonal showed some reactivity toward unreduced or reduced core protein. The core protein of the medium-derived proteoheparan sulfate did not bind to B3/25. The epitopes for these monoclonal antibodies are not known, but they are probably oligosaccharides (14, 15), which may be slightly different among various transferrin receptors (13) as well as in the proteoheparan sulfate core protein. In the latter case, the glycoprotein-type oligosaccharides may be close to the heparan sulfate-attachment region (3) and thereby shielded and inaccessible to the monoclonal antibodies.

The core protein of proteoheparan sulfate isolated from confluent fibroblasts appears to have the same subunit composition (M_r 2 × 90,000) as the transferrin receptor, binds to transferrin, and is recognized by a receptor-specific monoclonal antibody. These findings suggest that the core polypeptides of the proteoglycan and the receptor may be identical or closely similar. Like the transferrin receptor (2), the proteoglycan is probably anchored to the plasma membrane via a hydrophobic peptide portion. The proteoglycan, which may not bind to transferrin *in situ*, could be converted to a functional receptor by limited proteolysis or by the action of a heparan sulfate-degrading endo-β-glucuronidase (16, 17). It has been observed previously that growing cells degrade and shed heparan sulfate into the medium during the G_2 phase of the cell cycle (18). Thus, rapidly dividing cell populations contain less heparan sulfate than quiescent ones. Conversely, proliferating cells express more functional transferrin receptor at their surface than do nongrowing cells (2).

Table 3. Binding of monoclonal antibodies to [^{3}H]leucine-labeled proteoheparan sulfates and derivatives thereof

Proteoheparan sulfate from	Treatment	Monoclonal antibody	Binding to protein A % of added ^{3}H
Cell	None	B3/25	3
Cell	None	OKT9	4
Cell	None	L5/1	3
Cell	HS lyase	B3/25	97
Cell	HS lyase	OKT9	43
Cell	HS lyase	L5/1	6
Cell	HS lyase + R/A	OKT9	30
Medium	None	B3/25	18
Medium	None	OKT9	7
Medium	None	L5/1	8
Medium	HS lyase	B3/25	18

Treatments with heparan-sulfate (HS) lyase and reduction and alkylation (R/A) were performed.

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