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Endocytosis of Sulphated Proteoglycans by Cultured Skin Fibroblasts

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1. Human skin fibroblasts internalize homologous sulphated proteoglycans by adsorptive endocytosis. Endocytosis rate is half maximal when the concentration of the proteoglycans is 0.1 nM. At saturation, a single fibroblast may endocytose up to 8×10^6 proteoglycan molecules/h. 2. The kinetics of proteoglycan binding to the cell surface suggest the presence of 6×10^5 high-affinity binding sites per cell. The bulk of sulphated proteoglycans associates to low-affinity binding sites on the cell surface. 3. Glycosaminoglycans and other anionic macromolecules inhibit endocytosis of sulphated proteoglycans non-competitively. The lack of interaction of glycosaminoglycans with the cell-surface receptors for sulphated proteoglycans suggests that the protein core of proteoglycans is essential for binding to the cell surface. 4. The effects of trypsin, cell density, serum concentration and medium pH on endocytosis and degradation of endocytosed sulphated proteoglycans is described. 5. A comparison of the number of the high-affinity binding sites and the number of molecules endocytosed with respect to time suggests a recycling of the proteoglycan receptors between the cell surface and the endocytotic vesicles and/or the lysosomes.

Cultured human skin fibroblasts synthesize proteoglycans that are mainly secreted into the medium. Up to 30% of the newly formed proteoglycans, however, become associated with the cell surface and up to 20% may stay within the cells, where they are rapidly degraded (Neufeld & Cantz, 1973; Kresse *et al.*, 1975). Degradation of the carbohydrate side chains of proteoglycans appears to take place only within lysosomes. Before degradation the proteoglycans of the different compartments have therefore to be transferred to the lysosomes. Macromolecules from the cell surface and the exterior are internalized by cells via endocytosis. Endocytosis becomes a selective and highly efficient uptake mechanism if macromolecules are provided with a recognition marker that interacts with cell-surface receptors.

Previous studies in arterial fibroblasts showed that sulphated proteoglycans become internalized by adsorptive endocytosis, suggesting the involvement of specific binding (Kresse *et al.*, 1975). The present study extends these findings and provides information about (*a*) the interaction of sulphated proteoglycans with the cell surface of human skin fibroblasts, (*b*) the endocytosis and degradation of sulphated proteoglycans and (*c*) the effect of cell-culture variables and of macromolecules on these processes.

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Materials and Methods

Materials

Sodium [^{35}S]sulphate (sp. radioactivity 5 Ci/mg of S) and [^{35}S]heparin (37 mCi/g) were obtained from Amersham Buchler, Braunschweig, Germany; dextran, dextran sulphate, DNA and polygalacturonic acid were from Serva, Heidelberg, Germany; [^{3}H]heparan sulphate (from bovine aorta), hyaluronate (from human umbilical cord), dermatan sulphate (from calf skin) and chondroitin sulphate (from bovine nasal cartilage) were a gift from Dr. U. Klein of this Institute.

Methods

Cell culture. Human skin fibroblasts and bovine arterial fibroblasts (smooth-muscle cells) were grown in 75 cm^2 Falcon plastic flasks (LS Labor Service, München, Germany) in modified Eagle's minimum essential medium (Eagle, 1959), supplemented with 10% (v/v) foetal calf serum (LS Labor Service), non-essential amino acids, penicillin and streptomycin, as previously described (Cantz *et al.*, 1972).

Preparation of [^{35}S]proteoglycans from fibroblast secretions. Confluent cultures in Falcon flasks were incubated in the presence of 15 ml of serum-free medium [Gorham & Waymouth (1965) modified as

described by Fratantoni *et al.* (1969)] and 7 μCi of [^{35}S]sulphate/ml for 5 days. Medium was then removed, dialysed, concentrated and chromatographed on Sephadex G-200 (Pharmacia, Freiburg, Germany) as previously described (Kresse *et al.*, 1975). The ^{35}S-labelled uronic acid-containing material excluded from gel was used for endocytosis studies. The specific radioactivity of the [^{35}S]proteoglycans varied between 5×10^6 and 36×10^6 c.p.m./μmol of glucuronic acid. Rechromatography on a Sepharose 2B column calibrated with proteoglycan preparations of defined molecular weight revealed a mean molecular weight of about 1×10^5. Concentrations of [^{35}S]proteoglycans are given as disaccharide units calculated from glucuronic acid determination. For calculation of proteoglycan molarity the equivalence of 1 μmol of proteoglycan-bound disaccharide to 1 mg of proteoglycan (protein content about 40%) was assumed.

Determination of [^{35}S]proteoglycan endocytosis. Confluent monolayer cultures were prepared in 21 cm^2 Falcon dishes (LS Labor Service). Unless otherwise stated, cultures were incubated for 7h with 1.5ml of medium containing [^{35}S]proteoglycans in a concentration of 5nmol of disaccharide/ml. The dishes were kept under a gas phase of CO_2/air (19:1), at pH7.1. Determination of endocytosis, adsorption and degradation of [^{35}S]proteoglycans was exactly as described by Kresse *et al.* (1975). The rate of endocytosis was measured at non-saturating [^{35}S]proteoglycan concentrations and expressed as clearance rate, giving the volume of medium (in μl) cleared from [^{35}S]proteoglycans/h per mg of cell protein; 2.5×10^6 cells corresponded to 1mg of cell protein. Chondroitin [^{35}S]sulphate-rich proteoglycan was prepared from bovine nasal cartilage incubated *in vitro* by extraction with 4M-guanidinium chloride and caesium chloride-density-gradient centrifugation (Sajdera & Hascall, 1969). [^{35}S]Glycosaminoglycan chains were prepared from [^{35}S]proteoglycans and chondroitin [^{35}S]sulphate proteoglycan by treatment with 0.15M-NaOH at 37°C for 4h, followed by neutralization and dialysis against 0.15M-NaCl. Glucuronic acid was determined as described by Bitter & Muir (1962) and cell protein by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Cells were counted in a Coulter counter ZF (Coulter Electronics, Krefeld, Germany). Radioactivity was measured in a liquid-scintillation spectrometer (Packard, model B 2450), with Unisolve 1 (Zinsser, Frankfurt, Germany).

Results

Kinetics of [^{35}S]proteoglycan endocytosis

Cultured human skin fibroblasts synthesize and secrete proteoglycans containing dermatan sulphate, chondroitin sulphate and heparan sulphate as glycosaminoglycan components in the proportions 83:11:6 (Kresse *et al.*, 1975). On incubation of human skin fibroblasts for up to 5 days in the presence of [^{35}S]proteoglycans isolated from the secretions of these cells, the [^{35}S]proteoglycans associate with the cell surface, from which they can be solubilized by trypsin, and become internalized by the cells. The endocytosis is non-linear with time and appears to stop when 62% of added [^{35}S]proteoglycans are internalized (Fig. 1). The endocytosed [^{35}S]proteoglycans are transferred into the lysosomes, where they become rapidly degraded. The intracellular pool of macromolecular [^{35}S]glycosaminoglycans reaches a maximum between 24 and 48h, when endocytosis and degradation are in equilibrium. After 48h the rate of degradation exceeds that of endocytosis. The non-linear kinetics of endocytosis may be due to the depletion of the medium from [^{35}S]proteoglycan and/or the dilution of [^{35}S]proteoglycans in the medium by unlabelled proteoglycans secreted by the fibroblasts. The latter possibility appears to be very

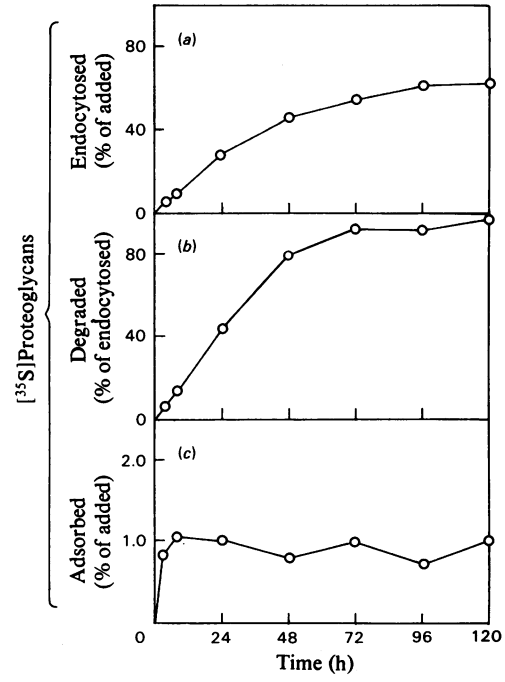

Fig. 1. *Time-dependence of [^{35}S]proteoglycan endocytosis* Human skin fibroblasts were incubated in the presence of 5nmol of [^{35}S]proteoglycan disaccharide/ml of medium. Endocytosis (*a*), degradation (*b*) of endocytosed [^{35}S]proteoglycans and adsorption (*c*) were determined after incubation for 4–120h. The mean cellular protein content was 0.43 (0.36–0.53) mg/dish.

unlikely, since the endocytosis rate of [^{35}S]proteoglycans was not measurably affected by conditioning the medium for up to 3 days. The apparent ceasing of endocytosis after 120h, when 40% of the added [^{35}S]proteoglycans are still present in the medium, indicates that only a part of the [^{35}S]proteoglycans is provided with structural properties required for endocytosis and/or that re-utilization of the liberated [^{35}S]sulphate for formation of [^{35}S]proteoglycans destined for secretion is in equilibrium with endocytosis. No evidence for a selective or prevalent endocytosis of individual proteoglycan types was obtained. During the whole incubation period the amount of the added [^{35}S]proteoglycans associated with the cell surface remains constant.

Endocytosis as a function of [^{35}S]proteoglycan concentration shows saturation kinetics (Fig. 2). In five experiments, fibroblasts internalized up to 0.7–5.3nmol of disaccharide/h per mg of cell protein (mean 3.5) at saturation, indicating that a single cell may endocytose up to 2pmol of disaccharide/h (approx. 8×10^6 proteoglycan molecules/h). The K_{uptake} values (concentration of [^{35}S]proteoglycans in the medium at half the maximum rate of endocytosis) varied from 10.7 to 89nmol of disaccharide/ml of medium (mean value 39.4nmol of disaccharide/ml, corresponding to approx. 0.4nM-[^{35}S]proteoglycan). The rate of degradation is independent of extracellular [^{35}S]proteoglycan concentration. In contrast with endocytosis, adsorption of [^{35}S]proteoglycans shows no dose-dependent saturation. Under our experimental conditions [^{35}S]proteoglycans equivalent to up to 17nmol of disaccharide were bound/mg of cell protein (approx. 0.8nmol of disaccharide per cm^2 growth area). At low proteoglycan concentrations (up to 30nmol of disaccharide/ml) high-affinity binding was observed. When the ratio of bound versus free [^{35}S]proteoglycans was plotted against bound [^{35}S]proteoglycans (Scatchard *et al.*, 1950), binding of 0.15 and 0.25nmol of disaccharide/mg of cell protein (two independent experiments) to high-affinity sites is observed (Fig. 3). This corresponds to approx. 6×10^5 high-affinity binding sites/fibroblast. The amount of radioactivity bound at low concentrations did not allow us to discriminate between saturable and/or non-saturable binding.

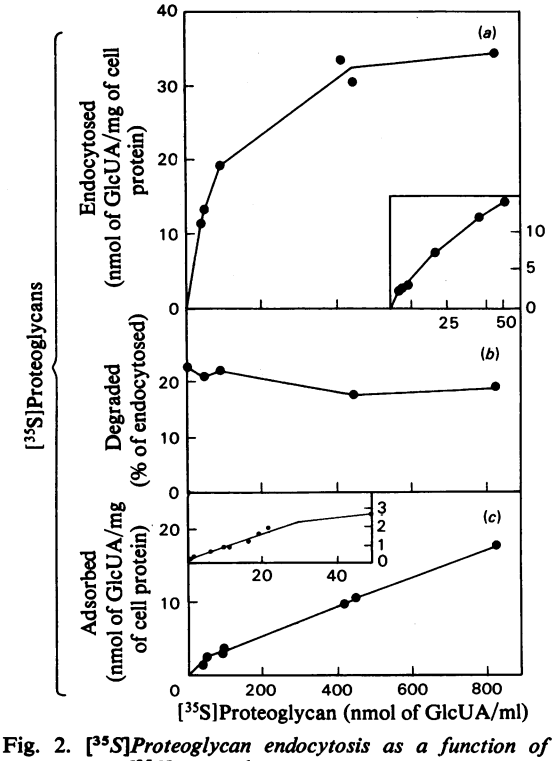

Fig. 2. [^{35}S]*Proteoglycan endocytosis as a function of* [^{35}S]*proteoglycan concentration*
Fibroblasts were incubated in the presence of 4.4–830nmol of [^{35}S]proteoglycan disaccharide/ml. After 7h the endocytosis (*a*), degradation (*b*) and adsorption (*c*) of [^{35}S]proteoglycans were measured. The mean cellular protein content was 0.35 (0.31–0.40) mg/dish.

Effect of trypsin treatment of fibroblasts on endocytosis of [^{35}S]*proteoglycans*

Confluent fibroblast cultures were treated with trypsin and seeded into new dishes without splitting, i.e. at the initial density of the confluent cultures. At various times after trypsin treatment endocytosis of [^{35}S]proteoglycans was assayed. As Fig. 4 shows, trypsin does not seem to destroy the ability of cells to

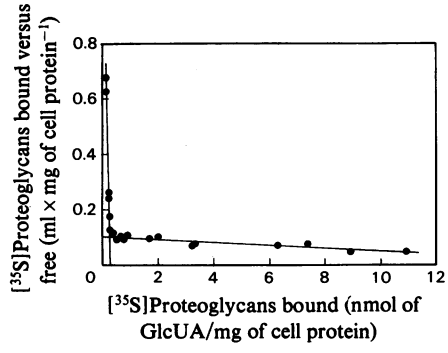

Fig. 3. *Scatchard plot of the binding of* [^{35}S]*proteoglycans to human skin fibroblasts*
Cells were incubated in the presence of 0.2–200nmol of [^{35}S]proteoglycan disaccharide/ml of medium. After 7h, [^{35}S]proteoglycans in the medium and those bound to the cell surface were measured.

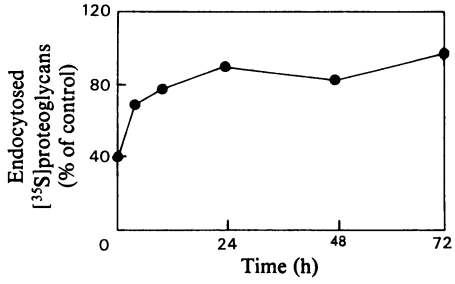

Fig. 4. *Effect of trypsin treatment on [35S]proteoglycan endocytosis*
After trypsin treatment at zero time, endocytosis during a 7h period was assayed up to 72h; 5nmol of proteoglycans was added/ml of medium. The mean cellular protein content was 0.38mg/dish.

endocytose [35S]proteoglycans. During a 7h period directly after trypsin treatment, the endocytosis rate is decreased to 40% of control values and reaches almost normal values within 24h.

Effect of cell density, serum concentration and pH on endocytosis of [35S]proteoglycans

Cells were seeded at densities ranging from 2000 to 30 000 cells/cm², and after incubation for 48h, [35S]proteoglycan endocytosis was assayed. At cell densities below apparent confluency ($<20\times10^3$ cells/cm²) cells endocytose [35S]proteoglycans at a lower rate than do confluent cultures, suggesting that endocytosis is decreased during the exponential growth phase (Fig. 5). This was confirmed by an experiment in which cells were seeded at a concentration of 15 000 cells/cm² and endocytosis was assayed after 2–19 days. The endocytosis rate was maximal after 10 days, when maximal cell density was reached. In both experiments the rate of degradation was independent of cell density, and adsorption was maximal in sparse cultures, where the surface/cell ratio is maximal. Foetal calf serum in concentrations between 0 and 20% has no influence on the adsorption, endocytosis and degradation of [35S]proteoglycans.

The pH-dependence of endocytosis of [35S]proteoglycans was assayed by adjusting the medium pH to 6.8–8.0 with various organic non-volatile buffers (Eagle, 1971). Whereas endocytosis is maximal around pH7.4, degradation is inversely related to the pH within the pH range 6.8–8.0 (Fig. 6).

Inhibition of [35S]proteoglycan endocytosis by macromolecules

Non-competitive inhibition of [35S]proteoglycan endocytosis was observed in the presence of various

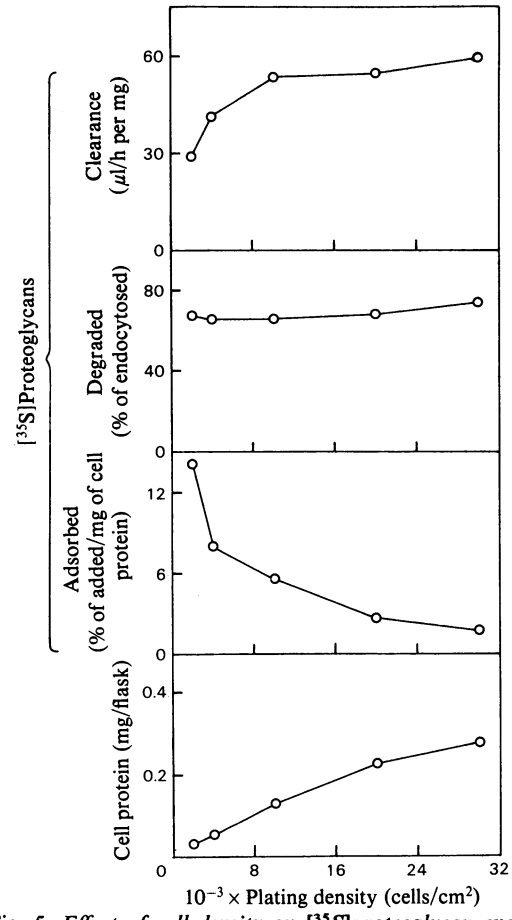

Fig. 5. *Effect of cell density on [35S]proteoglycan endocytosis*
Cells were seeded at densities ranging from 2000 to 30000 cells/cm². After incubation for 48h, endocytosis of [35S]proteoglycans was assayed under standard conditions (see under 'Methods').

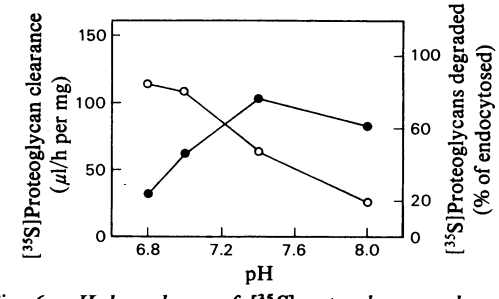

Fig. 6. *pH-dependence of [35S]proteoglycan endocytosis*
Media with pH values between 6.8 and 8.0 were prepared with non-volatile organic buffers. Endocytosis (●) and degradation (○) of [35S]proteoglycans were assayed during a 24h incubation period.

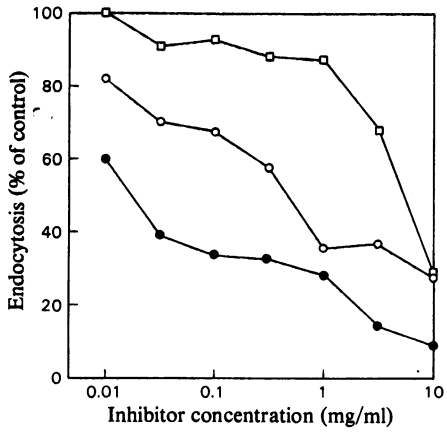

Fig. 7. *Inhibitory effect of polyanionic macromolecules on [^{35}S]proteoglycan endocytosis*
Endocytosis of [^{35}S]proteoglycans was assayed in the presence of 0.01–10 mg/ml of chondroitin sulphate (□), heparin (○) and dextran sulphate (●).

Table 1. *Concentration for 50% inhibition of [^{35}S]proteoglycan endocytosis by macromolecules*
The following compounds were non-inhibitory: Dextran, Dextran T200 and glycogen.

Compound	Concentration (mg/ml)
Dextran sulphate	0.023
Heparin	0.46
Polygalacturonic acid	0.95
Dermatan sulphate	2.5
Proteoglycan subunit	2.9
Chondroitin sulphate	5.2
DNA	5.2

glycosaminoglycans, DNA and polygalacturonic acid (Fig. 7 and Table 1). The degree of inhibition appears to be related to the negative charge of the macromolecules. Inconsistent inhibition was observed in the presence of chondroitin sulphate proteoglycan subunits from bovine nasal cartilage. Dextrans of various molecular size and glycogen produced no inhibition. Competitive inhibition was only observed in the presence of proteoglycan preparations prepared from the secretions of bovine arterial fibroblasts. Adsorption of [^{35}S]proteoglycans to the cell surface was not affected in the presence of the various non-competitive inhibitors.

Adsorption and endocytosis of glycosaminoglycan chains

Whereas [^{35}S]heparin, [^{3}H]heparan sulphate and chondroitin [^{35}S]sulphate inhibited non-competitively the [^{35}S]proteoglycan endocytosis (Table 1), these polysaccharide chains were virtually non-endocytosed. The amount of glycosaminoglycan chains adsorbed to the cell surface, however, was similar to that for [^{35}S]proteoglycans. At 3 μmol of disaccharide/ml of medium, between 10 and 50 nmol of disaccharides were bound per mg of cell protein (Fig. 8). The endocytosis rate of [^{35}S]glycosaminoglycan chains prepared from [^{35}S]proteoglycans secreted by fibroblasts was less than 1% of that of [^{35}S]proteoglycans.

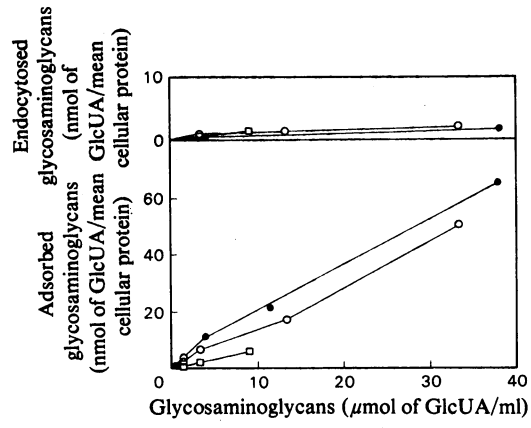

Fig. 8. *Endocytosis and adsorption of glycosaminoglycan chains*
Human skin fibroblasts were incubated in the presence of up to 38 μmol of chondroitin [^{35}S]-sulphate disaccharide units/ml (○), 33 μmol of [^{35}S]heparin disaccharide units/ml (●) and 9 μmol of [^{3}H]heparan sulphate disaccharide units/ml (□). Endocytosis and adsorption was assayed after a 7 h incubation period. The mean cellular protein content was 0.184 mg/dish.

Discussion

The kinetics of [^{35}S]proteoglycan endocytosis confirm previous results obtained with cultured bovine arterial fibroblasts (Kresse *et al.*, 1975). The endocytosis exhibits clear saturation kinetics. The affinity of [^{35}S]proteoglycans to the cell-surface receptors is about two orders of magnitude higher than that reported for the affinity of lysosomal enzymes to their cell-surface receptors in fibroblasts (von Figura & Kresse, 1974; Kaplan *et al.*, 1977; Sando & Neufeld, 1977; Ullrich *et al.*, 1978), whereas the number of molecules endocytosed/h per cell are remarkably similar for lysosomal enzymes and proteoglycans (von Figura, 1977). The kinetics of [^{35}S]proteoglycan binding to the cell surface suggest binding to low- and high-affinity binding sites. The low-affinity binding sites appear to be non-saturable.

Under our experimental conditions, binding of up to 5×10^{12} proteoglycan molecules per cm^2 surface was observed without any indication of saturable binding. The simplest explanation would be that sulphated proteoglycans associate with the cell surface as multilayers, owing to self-aggregation, rather than as a monolayer to low-affinity cell-surface receptors. A single cell that internalizes up to 8×10^6 proteoglycan molecules/h bears approx. 6×10^5 high-affinity binding sites. Assuming that endocytosis of sulphated proteoglycans requires binding to these high-affinity binding sites, that one binding site is used for endocytosis of one proteoglycan molecule and that adsorptive endocytosis of sulphated proteoglycans is accomplished as a proteoglycan–receptor complex, the half-life of the cell-surface receptor would be less than 3min at the maximal rate of endocytosis. Since $V_{max.}$ of endocytosis is maintained over a period of at least 24h, replenishment of cell-surface receptors for sulphated proteoglycan only by newly synthesized receptors is highly improbable. The high turnover rate of the proteoglycan receptor suggests a cellular mechanism that allows recycling of receptors between endocytotic vesicles and/or lysosomes and the plasma membrane. Recycling of cell-surface receptors has been postulated for immunoglobulin (Schneider *et al.*, 1977) and low-density-lipoprotein receptors (Anderson *et al.*, 1977).

Trypsin treatment decreased endocytosis of proteoglycans only for an initial period of 24h. This may indicate that the proteoglycan receptors are trypsin-resistant. Recycling of proteoglycan receptors, however, could easily replace trypsin-destroyed receptors within a period that is short compared with that required for determination of proteoglycan endocytosis. Diminished endocytosis rate during exponential growth phase as observed for sulphated proteoglycans has been reported for uptake of horseradish peroxidase by HeLa cells (Kaplan, 1976). Whereas the endocytosis rate of sulphated proteoglycans is maximal at pH7.4, that of lysosomal enzymes is inversely related to the pH between 6.8 and 8.0 (K. von Figura, unpublished work). The inhibitory effect of alkaline medium pH on intracellular degradation of endocytosed sulphated proteoglycans corresponds to the previously observed intralysosomal storage of [^{35}S]glycosaminoglycans in fibroblasts incubated at pH values above 7.4 (Lie *et al.*, 1972). The non-competitive inhibitory effect of negatively charged macromolecules such as glycosaminoglycans on endocytosis of sulphated proteoglycans suggests that these macromolecules do not interact with the cell-surface receptor for sulphated proteoglycans. It seems that adsorption of negatively charged macromolecules to the cell surface buries a part of proteoglycan receptors. Self-aggregation of sulphated proteoglycans on the cell surface and interaction of the sulphated proteoglycans with the remaining receptors appears to be unaffected. The self-aggregation of sulphated proteoglycans on the cell surface can be attributed to the glycosaminoglycan part of the proteoglycans, since similar amounts of free glycosaminoglycan chains and proteoglycan-bound glycosaminoglycan chains adsorb to the cell surface.

The lack of interaction of glycosaminoglycan chains with the cell-surface receptors for sulphated proteoglycans suggests that proteoglycans interact with the cell-surface receptors either via their protein core or via glycosaminoglycan side chains that have a conformation not maintained by free glycosaminoglycan chains. The final proof for this hypothesis, however, would be the demonstration of competitive inhibition of sulphated proteoglycan endocytosis by glycosaminoglycan-free protein-core molecules.

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