Heparan sulphate-degrading endoglycosidase in liver plasma membranes

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An endoglycosidase is described in isolated liver plasma membranes that brings about a rapid and selective degradation of membrane-associated heparan sulphate, pre-labelled biosynthetically with $\text{Na}_2^{35}\text{SO}_4$. The enzyme attacked mainly the polysaccharide chains of a hydrophobic membrane proteoglycan and it had little effect on a proteoglycan that could be displaced from the membranes with 1.0 M-NaCl. The highest activity was measured in the pH range 7.5–8.0, and the enzyme was almost completely inhibited below pH 5.5. Breakdown of susceptible polysaccharide chains was fast, being complete in 20–30 min. The major oligosaccharide fraction (M_r approx. 6000) produced by the enzyme was considerably smaller than the intact heparan sulphate chains. Enzyme activity was retained in membranes solubilized in 1% (v/v) Triton X-100. The high pH optimum and plasma-membrane association distinguish this enzyme from other heparan sulphate-degrading endoglycosidases that have acid pH optima and may be of lysosomal origin. A plasma-membrane endoglycosidase could modulate cellular interactions mediated by heparan sulphate, and/or release biologically active fragments of the polysaccharide from the cell periphery.

INTRODUCTION

Heparan sulphate proteoglycans are the major proteoglycans of rat liver plasma membranes [1,2]. By comparison with other cells and tissues, the liver proteoglycans are relatively small (M_r 73 000) and contain four short M_r -14 000 polysaccharide chains [3] that have a rather high content of ester sulphate groups [4]. Immunolocalization studies have confirmed that the proteoglycans are associated with the hepatocyte surface membrane, principally in the sinusoidal domain, but they are also found in endosomes, lysosomes and the Golgi apparatus [5].

Hepatocyte plasma membranes contain two populations of proteoglycan sulphates. One of these is hydrophobic and is presumed to be intercalated into the lipid bilayer [1]. The lipophilic domain may be composed of a sequence of hydrophobic amino acids, or a phosphatidylinositol moiety covalently attached to the protein core [6]. A second group of proteoglycans are associated with cell-surface receptors. In liver membranes and primary hepatocytes the receptors interact with the heparan sulphate chains, and this interaction is disrupted by hyperosmotic NaCl or heparin [2]. In an established hepatocyte cell line, a different membrane receptor has been found that seems to recognize the inositol phosphate moiety of the proteoglycan [6]. The receptor-associated proteoglycan is derived from the proteoglycan-phosphatidylinositol complex, probably by the action of a specific phospholipase C. The half-life of this hepatocyte proteoglycan is 4–6 h [7]. The vast majority of this material is lost via endocytosis and intracellular degradation, presumably in lysosomes. However, a small fraction of heparan sulphate enriched in the rare 2-*O*-sulphated glucuronate (GlcA-2S) units is released from the internalized proteoglycans and transported to the cell nucleus [8,9]. This polysaccharide has been implicated in cell growth control [6,9].

Metabolic studies carried out to date do not exclude the possibility that some degradation of proteoglycan may occur at the cell surface, accompanied by efficient uptake by the cells of the breakdown products. In 3T3 cells, for example, evidence has been obtained for the breakdown *in situ* of heparan sulphate proteoglycans in cell-substratum adhesion sites [10]. These proteoglycans play important roles in many cellular recognition and adhesion processes [11], and modulation of such interactions may require mechanisms for the pericellular degradation of the polysaccharide side chains or the protein core. In the present investigation we report on a novel enzyme activity in isolated liver plasma membranes that is responsible for a rapid, selective, breakdown of heparan sulphate chains.

MATERIALS AND METHODS

Materials

$\text{Na}_2^{35}\text{SO}_4$ (sp. radioactivity 1290 Ci/mmol) was obtained from Amersham International. DEAE-cellulose (DE52) came from Whatman. Sepharoses CL-4B and CL-6B, Sephadex G-100 and octyl-Sepharose were from Pharmacia Fine Chemicals. Bio-Gel P6 (200–400 mesh) was from Bio-Rad Laboratories. Guanidinium chloride was obtained from Fluka A.G., Buchs, Switzerland, and purified by filtration through activated charcoal in our laboratory before use. Pronase (from *Streptomyces griseus*) was from Boehringer Mannheim, and chondroitin sulphate, heparin and bovine serum albumin were all from Sigma. Optiphase 'Safe' scintillation cocktail came from L.K.B. All other chemicals used were of AnalaR quality.

Radiolabelling and preparation of liver plasma membranes

Two adult rats were both injected via the caudal vein with 0.8 ml of 0.9% NaCl containing 2.5 mCi of

$\text{Na}_2^{35}\text{SO}_4$. After 3 h the rats were killed, and the livers were removed and homogenized together with four unlabelled livers. Plasma membranes were prepared from a low-speed pellet of the liver homogenate as described previously [12] and stored in 8% (w/v) sucrose at -20°C .

Solubilization of plasma membranes

Plasma membranes suspended in 8% sucrose were centrifuged (10 500 *g* for 5 min), and the membrane pellet was resuspended in 4.0 M-guanidinium chloride, pH 5.4, containing 1% (v/v) Triton X-100, 100 μg of bovine serum albumin/ml, 100 μg of soya-bean trypsin inhibitor/ml, 1 mM-phenylmethanesulphonyl fluoride and 10 mM-*N*-ethylmaleimide. The solution was incubated at 4°C with continuous gentle agitation for 1 h. After this time, no particulate material was visible and all the proteoglycan was in solution. In some experiments, proteoglycans were extracted in a similar manner but without the 4.0 M-guanidinium chloride. This procedure also completely solubilized the membranes. These extracts, after a further centrifugation step, were applied directly to Sepharose CL-6B or dialysed against 50 mM-sodium phosphate buffer, pH 5.0, containing 6 M-urea and 1% Triton X-100 before chromatography on DEAE-cellulose.

Separation of plasma-membrane proteoglycan pools

(a) **Receptor-bound proteoglycans.** These were released with hyperosmotic salt as described by Hook and colleagues [2]. Membranes suspended in sucrose were pelleted by centrifugation (10 500 *g* for 5 min) in an MSE micro-centrifuge and resuspended in 1.0 M-NaCl/50 mM-sodium phosphate buffer, pH 5.0. Membranes were incubated for 1 h at 4°C , pelleted by centrifugation, and the supernatant was recovered carefully with a microsyringe. Proteoglycans in the membrane pellet were solubilized in 1% Triton X-100 with proteinase inhibitors as described above.

(b) **Release of heparan sulphate oligosaccharides by an endogenous endoglycosidase.** Enzyme-mediated release of heparan sulphate was carried out by incubating plasma membranes in 20 mM-sodium phosphate buffer, pH 7.6, containing 0.15 M-NaCl for 30–60 min at 37°C . The membranes were pelleted by centrifugation, and the clear supernatant was carefully removed. The membrane pellet was then dissolved in 1% Triton X-100. Incubations were also carried out in the presence of 100 μg of bovine serum albumin/ml or with one of the following proteinase inhibitors: 10 mM-EGTA, 10 mM-*N*-ethylmaleimide, 1 mM-phenylmethanesulphonyl fluoride, 100 μg of soya-bean trypsin inhibitor/ml and 0.2 μg of either pepstatin, chymostatin or leupeptin/ml.

Gel-filtration chromatography

This was carried out on Sepharose CL-6B (bed volume 115 cm $\times$ 1 cm) equilibrated in 4 M-guanidinium chloride/1% Triton X-100 containing 10 μg of bovine serum albumin/ml, pH 6.0. Fractions (1 ml) were collected at a flow rate of 3 ml/h. Sephadex G-100 (bed volume 75 cm $\times$ 1 cm) was eluted with 0.5 M-NaCl containing 1% Triton X-100 at a flow rate of 6.5 ml/h; 0.55 ml fractions were collected. These columns were pretreated with 1 mg each of heparin, chondroitin sulphate and bovine serum albumin. Bio-Gel P6 (200–400

mesh) in a column of 1.5 cm $\times$ 60 cm was eluted at a flow rate of 8 ml/h with 0.5 M- NH_4HCO_3 . The V_0 and V_t values for the gel filtration columns were determined with Blue Dextran and [^3H]glucosamine respectively.

DEAE-cellulose chromatography

A column of DEAE-cellulose DE-52 (8 cm $\times$ 1 cm) was equilibrated with 6 M-urea/1% Triton X-100/0.15 M-NaCl/50 mM-sodium phosphate buffer, pH 5.0. Samples of solubilized plasma membranes in the same solution were applied to the column, which was washed with 3 bed volumes of buffer before elution with a linear gradient from 0.15–0.8 M-NaCl superimposed on the basic equilibration solution; 2 ml fractions were collected at a flow rate of 14 ml/h.

Hydrophobic chromatography on octyl-Sepharose

An octyl-Sepharose column (5 cm $\times$ 0.6 cm) was washed thoroughly with 50 mM-sodium phosphate buffer, pH 5.0, containing 100 μg of bovine serum albumin/ml. Samples of plasma membranes dissolved in 1% Triton X-100/50 mM-sodium phosphate, pH 5.0, were applied to the column, by using sample volumes less than one-tenth of the column volume. Triton micelles containing intercalated hydrophobic proteoglycans bind immediately to the octyl-Sepharose [13]. The column was then eluted stepwise with 10 ml portions of the following solutions: (1) 50 mM-sodium phosphate, pH 5.0; (2) 0.5 M-LiCl; (3) 0.5 M-LiCl containing 1 mg of heparin/ml; (4) 1% (w/v) SDS in 0.5 M-LiCl. Solutions 2, 3 and 4 were all buffered to pH 5.0 with 50 mM-sodium phosphate.

Pronase digestions

Membranes suspended in 10 mM-Tris/acetate buffer, pH 7.8, containing 1 M-NaCl and 1% Triton X-100 were incubated with Pronase (1 mg/ml) at 37°C for 24 h. Additional Pronase (0.5 mg/ml) was added after 18 h. After incubation, the digest was cooled at 4°C , and

Fig. 1. HNO_2 hydrolysis of ^{35}S -labelled macromolecules of hepatocyte plasma membranes

Pronase digests of hepatocyte plasma membranes were treated with HNO_2 (pH 1.5) and fractionated on a Bio-Gel P-6 column (1.5 cm $\times$ 60 cm). The flow rate was 8 ml/h, and 1 ml fractions were collected. ----, Untreated material; —, material treated with HNO_2 .

Fig. 2. Chromatography of ^{35}S -labelled heparan sulphate on DEAE-cellulose

Plasma membranes were dissolved in 4.0 M-guanidinium chloride/1 % Triton X-100, pH 6.0, and then dialysed against 6 M-urea/1 % Triton X-100/0.15 M-NaCl/50 mM-sodium phosphate, pH 5.0. The solution was applied directly to a DEAE-cellulose DE-52 column (8 cm $\times$ 1 cm) and fractionated with a linear NaCl gradient (0.15–0.8 M) in 6 M-urea/1 % Triton X-100. The flow rate was 14 ml/h, and 2 ml fractions were collected. Radioactivity in the major peak (fractions 45–70) was degraded by treatment with HNO_2 .

protein was precipitated by the addition of 0.1 vol. of ice-cold 100 % (w/v) trichloroacetic acid. The solution was mixed carefully and left at 4 °C for 1 h. A precipitate was removed by centrifugation (800 g for 5 min). The supernatant was dialysed against a large excess of distilled water and then concentrated to a small volume by rotary evaporation.

Alkaline elimination

Samples of heparan sulphate proteoglycan were taken up in 50 mM-NaOH containing 1 M- NaBH_4 and incubated for 18 h at 37 °C. After neutralization with 1 M-acetic acid, samples were chromatographed on Sepharose CL-6B or Sephadex G-100.

Hydrolysis with HNO_2

^{35}S -labelled polysaccharide in 50 μl of water was mixed with 0.5 ml of freshly prepared HNO_2 , pH 1.5 [14]. After 10 min at room temperature, the acid was neutralized by addition of 0.25 vol. of 2 M- Na_2CO_3 and the samples were fractionated on a Bio-Gel P6 column.

RESULTS

Identification of ^{35}S -labelled heparan sulphate in liver plasma membranes

Rat liver plasma membranes isolated 3 h after injection of the animal with [^{35}S]sulphate were digested with Pronase, and the soluble polysaccharides remaining after precipitation with trichloroacetic acid were examined. In different membrane preparations, recovery of [^{35}S]-

Fig. 3. Sepharose CL-6B gel filtration of native and Pronase-digested heparan sulphate proteoglycan

^{35}S -labelled plasma membranes were dissolved in the column equilibration buffer (4.0 M-guanidinium chloride/1 % Triton X-100, pH 6.0), applied to a Sepharose CL-6B column (1 cm $\times$ 115 cm) and eluted at a flow rate of 3 ml/h. Pronase digestion was carried out on membranes taken up directly in 10 mM-Tris/acetate, pH 7.8, containing 1 M-NaCl, 1 % Triton X-100 and Pronase (5 mg/ml). Samples were incubated for 8 h at 37 °C and then applied directly to the Sepharose CL-6B column. —, Undigested membrane proteoglycans; ----, Pronase-digested samples.

sulphate in the soluble fraction was consistently high (98–100 %), and 90–95 % of this material was degraded by low-pH HNO_2 (Fig. 1), indicating that the major metabolically labelled component was heparan sulphate; the remaining 5–10 % of labelled material was not identified.

Analysis of heparan sulphate proteoglycans

^{35}S -labelled proteoglycans in liver plasma membranes were completely solubilized in 1 % Triton X-100/4.0 M-guanidinium chloride (see the Materials and methods section). The extract was dialysed against 1 % Triton X-100/6 M-urea and analysed by NaCl-gradient elution on DEAE-cellulose (Fig. 2). A small amount of label was eluted at 0.35 M-NaCl, followed by the main ^{35}S -labelled proteoglycan peak, which emerged at about 0.5 M-NaCl.

Analysis of the proteoglycan by gel filtration on Sepharose CL-6B (Fig. 3) yielded a major large component (K_{av} , 0.21) and a smaller minor species (K_{av} , $\sim$ 0.5). Pronase digestion decreased the size of the proteoglycan to yield a main component of K_{av} , 0.44 plus a minor, higher-molecular-size, species of K_{av} , 0.25 (Fig. 3). Alternatively, treatment with alkaline borohydride, which should liberate free polysaccharide chains, yielded a single symmetrical peak (results not shown), which was eluted at the same position as the main component generated by Pronase digestion (i.e. K_{av} , of 0.44).

Fig. 4. Effect of pH on the release of ³⁵S-labelled heparan sulphate from hepatocyte plasma membranes

Plasma membranes in 8% (w/v) sucrose were pelleted at 4 °C and then taken up in 100 μ l of buffered solutions covering the pH range 3.5–8.0 (see the Materials and methods section for details) and incubated at 37 °C for 30 min. Released ³⁵S-labelled heparan sulphate was separated from membrane-bound material by centrifugation at 10 500 *g* for 5 min at 4 °C, and radioactivity was determined in both the pellet and supernatant. Released ³⁵S-labelled heparan sulphate is expressed as a percentage of the total radioactivity in the sample.

These results show that a single-molecular-size group of ³⁵S-labelled heparan sulphate chains are present as proteoglycan in liver plasma membranes. The data are similar to those reported by other workers [1–3].

Enzyme-mediated release of plasma-membrane heparan sulphate

Plasma membranes were incubated at 37 °C for 30 min in 0.15 M-NaCl over the pH range 3.5–8.0. The loss of radiolabelled polysaccharide from the membranes showed a pronounced pH-dependence, consistent with an enzyme-mediated process, being minimal below pH 5.5 and approaching a maximum value at pH 7.5–8.0 (Fig. 4). In the example shown, 38% of the polysaccharide was released, though with different membrane preparations this value ranged from 30 to 65% of the total membrane-associated material. Higher-pH solutions were avoided, in order to prevent the possible release of the extrinsic proteoglycan, which may be bound to a membrane receptor by ionic bonds [2]. To check that the low-pH conditions were not irreversibly inactivating the enzyme, a sample of membranes used in the experiment shown in Fig. 4 was preincubated at pH 5.0 for 30 min at 37 °C, and then transferred to pH 7.5. Release of radiolabel from these membranes (36% of the total ³⁵S) was similar to that in the membranes incubated directly at pH 7.5. Further investigations showed that the release process was

Fig. 5. Rate of release of ³⁵S-labelled heparan sulphate from plasma membranes

Plasma membranes were incubated at 37 °C in 100 μ l of 20 mM-sodium phosphate buffer, pH 7.5, containing 0.15 M-NaCl. At specified times the tubes were placed in crushed ice for 30 s, and membranes were pelleted by centrifugation (10 500 *g*) at 4 °C. Released heparan sulphate was determined as described for Fig. 4.

Fig. 6. Comparison of gel-filtration profiles of membrane-associated and released ³⁵S-labelled heparan sulphate after incubation of plasma membranes at pH 7.5, 37 °C

Plasma membranes were incubated in 20 mM-sodium phosphate buffer, pH 7.5, containing 0.15 M-NaCl (100 μ l) for 30 min at 37 °C. Membrane-bound and soluble fractions were separated by centrifugation, and each was taken up in 1 ml of 4.0 M-guanidinium chloride/1% Triton X-100, pH 6.0, and fractionated on Sepharose CL-6B as described in Fig. 3. —, Membrane-bound heparan sulphate; ----, soluble heparan sulphate.

unaffected by a variety of proteinase inhibitors (see the Materials and methods section). The rate of release of susceptible heparan sulphate from the plasma membranes was rapid and approached completion within 20–30 min (Fig. 5).

Fig. 7. Sephadex G-100 gel filtration of the heparan sulphate released by the plasma-membrane endoglycosidase

Membranes were incubated for 30 min under conditions described in Fig. 6. The soluble and membrane-associated heparan sulphates were prepared by centrifugation and chromatographed on Sephadex G-100 (75 cm $\times$ 1 cm) eluted in 0.5 M-NaCl/1 % Triton X-100. The flow rate was 6.5 ml/h, and 0.55 ml fractions were collected. —, Soluble heparan sulphate; ----, membrane-associated heparan sulphate.

Gel filtration of enzyme-solubilized and membrane-bound heparan sulphate

Gel filtration on Sepharose CL-6B showed that the radioactive material released from the plasma membranes at pH 7.5 contained a main peak of activity at K_{av} 0.73 (Fig. 6), which is considerably smaller than the intact heparan sulphate chains of the proteoglycan (K_{av} 0.44). The degraded fragments, which were sensitive to HNO_2 hydrolysis, thus represent heparan sulphate oligosaccharides. The ^{35}S -labelled components that remained bound to the incubated membranes comprised two species when fractionated on Sepharose CL-6B (Fig. 6), namely a major component identical in size with the intact proteoglycan (and therefore presumably undegraded), and a smaller minor fraction (K_{av} 0.44), which corresponded in size to the free heparan sulphate chains (see Fig. 3). When chromatographed on a Sephadex G-100 column, these membrane-bound proteoglycans and polysaccharides were eluted in the void-volume fraction (Fig. 7). In contrast, the released heparan sulphate oligosaccharides showed a clear bimodal distribution on Sephadex G-100 (Fig. 7), with the major fraction of radioactivity having a peak K_{av} of 0.6, indicating an average M_r of about 7000 [15]. The release from plasma membranes of these oligosaccharides clearly establishes the presence of a membrane-associated endoglycosidase.

The endoglycosidase is active in soluble form

To examine whether enzyme activity required intact plasma membranes, samples were dissolved in 100 μl of

Fig. 8. Degradation of heparan sulphate proteoglycans in detergent-solubilized membranes demonstrated by gel filtration on Sepharose CL-6B

Plasma membranes were taken up in 1 % Triton X-100/20 mM-sodium phosphate buffer, pH 7.5, containing 0.15 M-NaCl and incubated for 1 h at 37 °C. The preparation was then fractionated on Sepharose CL-6B (see the legend to Fig. 3).

Triton X-100 in 50 mM-sodium phosphate buffer, pH 7.5, and incubated at 37 °C for 1 h. After incubation, gel chromatography on Sepharose CL-6B identified three radioactive peaks in the solubilized preparation, one (K_{av} 0.21) corresponding to the whole proteoglycan, and two others with K_{av} values of 0.42 and 0.7 representing degradation products (Fig. 8). These degraded materials were identical in size with those formed after incubation of intact membranes (compare with the two most retarded peaks in Fig. 6). Therefore the enzyme is active in a soluble form, and it apparently continues to display the same restricted specificity (i.e. a significant fraction of the proteoglycan is undegraded) that is apparent in the intact membranes. Addition of heparin (1 mg/ml) completely inhibited the degradation of heparan sulphate in soluble membrane preparations, whereas chondroitin sulphate had little inhibitory activity (results not shown).

Endoglycosidase activity selectively degrades the polysaccharide chains of the hydrophobic heparan sulphate proteoglycan

(a) **Identification of hydrophobic and receptor-bound (i.e. extrinsic) proteoglycan.** Because the endoglycosidase did not degrade all the heparan sulphate chains, it was possible that the enzyme-susceptible polysaccharides were not randomly distributed amongst the liver proteoglycans, but were associated selectively with either the hydrophobic or the receptor-bound proteoglycans that have been demonstrated previously in liver membranes (see the Introduction). To assess this possibility, we had to establish that these two groups of proteoglycans were present in our membrane preparations.

^{35}S -labelled plasma membranes were solubilized in Triton X-100 at pH 5.0, conditions incompatible with endoglycosidase activity, and applied directly to octyl-

Fig. 9. Hydrophobic chromatography of plasma-membrane heparan sulphate proteoglycans

(a) Plasma membranes dissolved in 100 μ l of 1% Triton X-100/50 mM-sodium phosphate buffer, pH 5.0, were applied to an octyl-Sepharose column (5 cm $\times$ 0.6 cm). The column was then eluted sequentially and stepwise with 10 ml portions of the following: (i) 50 mM-sodium phosphate, pH 5.0; (ii) 0.5 M-LiCl; (iii) 0.5 M-LiCl plus heparin (1 mg/ml); (iv) 0.5 M-LiCl containing 1% SDS. Each step is indicated by an arrow. Solutions (ii)–(iv) were buffered to pH 5.0 with 20 mM-sodium phosphate. Fraction volume was 1 ml. (b) Membranes were incubated for 30 min at 4 $^{\circ}$ C in 100 μ l of 1.0 M-NaCl/50 mM-sodium phosphate buffer, pH 5.0, to displace proteoglycans bound to plasma-membrane receptors. The membranes were pelleted by centrifugation and the supernatant was carefully recovered. The membrane pellet was taken up in 1% Triton X-100/50 mM-sodium phosphate, pH 5.0; 200 μ l of this Triton-containing buffer was also added to the 1.0 M-NaCl supernatant containing the released proteoglycans. The supernatant and solubilized membrane pellet were then examined by hydrophobic chromatography. —, Membrane-associated (hydrophobic) proteoglycans; ----, 1.0 M-NaCl-released (receptor-bound) proteoglycans.

Fig. 10. Influence of plasma-membrane-associated endoglycosidase on the hydrophobic membrane proteoglycans

Plasma membranes were incubated at 37 $^{\circ}$ C for 30 min in conditions that were compatible with endoglycosidase activity (20 mM-sodium phosphate, pH 7.5, in 0.15 M-NaCl). The membranes were then separated by centrifugation, and the membrane-associated (a) and released (b) heparan sulphates were examined by hydrophobic chromatography as described in the legend to Fig. 9.

Sephacrose. The gel column was eluted sequentially and stepwise with 0.15 M-NaCl, 0.5 M-LiCl, heparin (1 mg/ml) and 1% SDS (see the Materials and methods section and the legend to Fig. 9). Two major peaks of radioactivity were observed, one in the 0.5 M-LiCl step and the other in the SDS step, representing 40% and 48% respectively of the applied 35 S radioactivity (Fig. 9a). The remaining 12% of the 35 S label was eluted in the heparin step. The hydrophobic proteoglycan is presumed to be that eluted with SDS, and the non-hydrophobic molecules eluted with 0.5 M-LiCl or heparin are considered to be the extrinsic species that are bound to receptors in the intact membranes.

A further experiment broadly substantiated these conclusions. Plasma membranes were incubated at 4 $^{\circ}$ C

for 30 min in 1.0 M-NaCl, pH 5.0. These conditions should displace the receptor-bound proteoglycans [2], but should inhibit the endoglycosidase and should not affect the membrane-anchoring domain of the hydrophobic proteoglycan. When the 1.0 M-NaCl-released ^{35}S -labelled materials were examined by hydrophobic chromatography (Fig. 9b), most of the radioactivity was eluted with 0.5 M-LiCl or washed through the column unretarded, and only 8% of the applied radioactivity was recovered in the SDS-eluted fraction. By contrast, the Triton-solubilized membranes that had been previously exposed to hyperosmotic NaCl contained mainly hydrophobic proteoglycans (eluted with SDS), though 20% of the applied label was eluted by heparin (Fig. 9b). Incubation of untreated membranes with inositol 2-phosphate (2 mM) or inositol hexakisphosphate (5 mM) did not displace any proteoglycan from these membranes [6].

(b) Enzyme-mediated release of the heparan sulphate chains of the hydrophobic proteoglycan. Plasma membranes were incubated under conditions that were compatible with the demonstrated endoglycosidase activity (pH 7.5 at 37 °C for 30 min). After centrifugation, the membrane pellet and supernatant fractions were analysed by hydrophobic chromatography as described above. The supernatant fraction, which contained the endoglycosidase-released heparan sulphate oligosaccharides (see Fig. 6), was essentially devoid of hydrophobic species, the major peak being eluted with 0.5 M-LiCl (Fig. 10b). Similarly hydrophobic chromatography of the membrane-associated fraction, which contained mainly undegraded proteoglycans (see Fig. 6), also yielded the major peak of radioactivity in the 0.5 M-LiCl step, and only a minor fraction of the sample (24%) was eluted with SDS (Fig. 10b). The proportion of hydrophobic proteoglycans remaining after endoglycosidase activity was only 12.5% of the total ^{35}S -labelled proteoglycans in the original membranes (this value is obtained by summation of the radioactivity values in Figs. 10(a) and 10(b) and then calculating the percentage of hydrophobic proteoglycan from the radioactivity in the SDS-eluted fraction in Fig. 10(a). Before endoglycosidase action, 48% of the proteoglycan in these membranes expressed hydrophobic properties (Fig. 9a). These results therefore strongly suggest that the endoglycosidase is degrading mainly the heparan sulphate chains belonging to the hydrophobic proteoglycan.

DISCUSSION

Several mammalian enzymes have been described that can achieve partial degradation of heparan sulphate [16–18]. Although these enzymes can differ in the required chemical properties of their polysaccharide substrates, they are mainly endo- β -glucuronidases that attack the glucuronosyl linkage in sequences of GlcA- β 1 $\rightarrow$ 4-GlcNAc [19–23]. An endo- β -glucuronidase that degrades heparin has been identified in mastocytomas [24]. The pH optimum of these enzymes is in the range 5.0–6.0, and they may be lysosomal in origin.

The liver membrane endoglycosidase described in the present paper can be distinguished from the foregoing enzymes in a number of ways, most conspicuously by its

high pH optimum (7.5–8.0) and the lack of activity below pH 5.5 (Fig. 4). It seems to be the first plasma-membrane enzyme to be described which has the capacity to degrade heparan sulphate. The pH/activity profile is similar to that of other rat liver plasma-membrane enzymes [25,26] and is compatible with a physiological function at the cell surface. However, we can only speculate on what that function might be. The enzyme could modify cell-cell and cell-biomatrix interactions that are mediated by heparan sulphate [27–29]. Conceivably, cell migration or 'cell-rounding' during mitosis could depend on the controlled hydrolysis of the polysaccharide chain [30].

An alternative view would be that the enzyme is inactive in whole plasma membranes. Activation may occur only after co-internalization with heparan sulphate proteoglycan in a primary endosome (preparation of isolated plasma membrane vesicles could achieve a similar effect). A fast-acting, high-pH-optimum, endoglycosidase could bring about a rapid partial depolymerization of the polysaccharide substrate before activity is suppressed by endosome acidification to pH 5.0, a process normally complete within 5 min of endosome formation [31]. Evidence for a role of such an enzyme in the degradation of internalized heparan sulphate has been found in granulosa cells [32], hepatocytes [7] and colonic carcinoma [33]. The release from endocytosed proteoglycan of the distinctive heparan sulphate chains that are transported to the hepatocyte nucleus, and which may possess a growth-regulatory role, occurs independently of endosome acidification [8]. These nuclear components could be formed by an endoglycosidase similar to that described here. Furthermore, oligosaccharides which resemble in molecular size those illustrated in Fig. 7 accumulate in lysosome-like organelles during the intracellular degradation of surface-membrane heparan sulphate in primary hepatocyte cell cultures [21]. These oligosaccharides were the products of an endoglucuronidase, but the cellular location of the enzyme was not established.

The mechanism of association of the enzyme with plasma membranes is unknown. Recent work (results not shown) has indicated that enzyme activity is lost when membranes are incubated in 0.4 M-NaCl (at this concentration of NaCl, the extrinsic proteoglycan is not displaced from the membranes). This finding suggests an ionic interaction with a plasma-membrane component, which could be the negatively charged heparan sulphate chains that are substrates for the enzyme. Curiously, we were unable to demonstrate heparan sulphate-degrading activity in the 0.4 M-NaCl extract of the membranes. The reasons for this require investigation.

The demonstrated selective mode of action of the plasma-membrane endoglycosidase (Figs. 6, 8 and 10) is intriguing. Selectivity might be related to the accessibility of substrate, with the polysaccharides of the enzyme-susceptible hydrophobic proteoglycan being freely available, whereas those of the extrinsic proteoglycan are shielded by their interaction with plasma-membrane receptors. On the other hand, only the polysaccharide chains of the hydrophobic proteoglycan may contain the sugar sequences recognized by the enzyme. Elucidation of the specificity of the liver membrane enzyme, its site of action in the cell and its mode of regulation should enhance our understanding of the molecular structure and biological function of heparan sulphate.

REFERENCES

1. Kjellén, L., Pettersson, I. & Höök, M. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 5371–5375
2. Kjellén, L., Oldberg, A. & Höök, M. (1980) *J. Biol. Chem.* **255**, 10407–10413
3. Oldberg, A., Kjellén, L. & Höök, M. (1979) *J. Biol. Chem.* **254**, 8505–8510
4. Gallagher, J. T. & Walker, M. (1985) *Biochem. J.* **230**, 665–674
5. Stow, J. L., Kjellén, L., Unger, E., Höök, M. & Farquhar, M. G. (1985) *J. Cell Biol.* **100**, 975–980
6. Ishihara, M., Fedarko, N. S. & Conrad, H. E. (1987) *J. Biol. Chem.* **262**, 4708–4716
7. Bienkowski, M. J. & Conrad, H. E. (1984) *J. Biol. Chem.* **259**, 12989–12996
8. Ishihara, M., Fedarko, N. S. & Conrad, H. E. (1986) *J. Biol. Chem.* **261**, 13575–13580
9. Fedarko, N. S. & Conrad, H. E. (1986) *J. Cell Biol.* **102**, 587–599
10. Lark, M. W. & Culp, L. A. (1984) *J. Biol. Chem.* **259**, 212–217
11. Gallagher, J. T., Lyon, M. & Steward, W. P. (1986) *Biochem. J.* **236**, 313–325
12. Evans, W. H., Flint, N. A. & Vischer, P. (1980) *Biochem. J.* **192**, 903–910
13. Carson, S. D. & Konigsberg, W. A. (1981) *Anal. Biochem.* **116**, 398–401
14. Shively, J. E. & Conrad, H. E. (1976) *Biochemistry* **15**, 3932–3942
15. Laurent, T. C., Tengblad, A., Thunberg, L., Höök, M. & Lindahl, U. (1978) *Biochem. J.* **175**, 691–701
16. Hook, M., Wasteson, A. & Oldberg, A. (1975) *Biochem. Biophys. Res. Commun.* **67**, 1422–1428
17. Klein, U., Kresse, H. & von Figura, K. (1976) *Biochem. Biophys. Res. Commun.* **69**, 158–164
18. Bar-Ner, M., Mayer, M., Schirmacher, V. & Vlodavsky, I. (1986) *J. Cell. Physiol.* **128**, 299–306
19. Oldberg, A., Heldin, C. H., Wasteson, Å., Busch, C. & Höök, M. (1980) *Biochemistry* **19**, 5755–5762
20. Oosta, G. M., Favreau, L. V., Beeler, D. L. & Rosenberg, R. D. (1982) *J. Biol. Chem.* **257**, 11249–11255
21. Kjellén, L., Pertoft, H., Oldberg, A. & Höök, M. (1985) *J. Biol. Chem.* **260**, 8416–8422
22. Nakajima, M., Imamura, T., Di Ferrante, N. & Nicolson, G. L. (1984) *J. Biol. Chem.* **259**, 2283–2290
23. Klein, U. & von Figura, K. (1979) *Hoppe-Seyler's Z. Physiol. Chem.* **360**, 1465–1471
24. Thunberg, L., Bäckström, G., Wasteson, Å., Robinson, H. C., Ögren, S. & Lindahl, U. (1982) *J. Biol. Chem.* **257**, 10278–10282
25. Evans, W. H., Hood, D. O. & Gurd, J. W. (1973) *Biochem. J.* **135**, 827–832
26. Evans, W. H. & Gurd, J. W. (1973) *Biochem. J.* **133**, 189–199
27. Schubert, D. & La Corbiere, M. (1985) *J. Cell Biol.* **100**, 56–63
28. Cole, G. J., Loewy, A. & Glaser, L. (1986) *Nature (London)* **320**, 445–447
29. Kraemer, P. M. & Tobey, R. A. (1972) *J. Cell Biol.* **55**, 713–717
30. Woods, A., Couchman, J. R., Johansson, S. & Höök, M. (1986) *EMBO J.* **5**, 665–670
31. Mellman, I., Fucho, R. & Helenius, A. (1986) *Annu. Rev. Biochem.* **55**, 663–700
32. Yanagishita, M. & Hascall, V. C. (1984) *J. Biol. Chem.* **259**, 10270–10283
33. Iozzo, R. V. (1987) *J. Biol. Chem.* **262**, 1888–1900

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