Structure and interactions of proteoglycans in the extracellular matrix produced by cultured human fibroblasts

Staffan JOHANSSON,*|| Klaus HEDMAN,† Lena KJELLÉN,‡ James CHRISTNER,§ Antti VAHERI† and Magnus HÖÖK§

*Department of Medical and Physiological Chemistry, University of Uppsala, S-751 23 Uppsala, Sweden, †Department of Virology, University of Helsinki, 00290 Helsinki 29, Finland, ‡Department of Medical and Physiological Chemistry, Swedish University of Agricultural Sciences, S-75123 Uppsala, Sweden, and §Connective Tissue Laboratory, Diabetes Research and Training Center, University of Alabama, Birmingham, AL 35294, U.S.A.

Subconfluent cultures of human embryonic skin fibroblasts were labelled with [³⁵S]sulphate for 3 days, after which cell-free extracellular matrix was isolated. A chondroitin sulphate proteoglycan (CSPG) and a heparan sulphate proteoglycan (HSPG) were purified from the matrix. Chromatography on Sepharose CL-2B gave peak K_{av} values of 0.35 and 0.38 respectively for the CSPG and the HSPG. The polysaccharide chains released from the two PGs were of similar size (K_{av} 0.50 on Sepharose CL-4B). Approx. 50% of the CSPG showed affinity for hyaluronic acid (HA). However, it differed immunologically from the HA-aggregating CSPG of human articular cartilage, and had a larger core protein (apparent molecular mass 290 kDa) than had the cartilage PG. Neither metabolically [³⁵S]sulphate-labelled PGs, isolated from the medium of fibroblast cultures, nor chemically ³H-labelled polysaccharides (HA, CS, HS and heparin) were incorporated into the extracellular matrix when added to unlabelled cell cultures. These results indicate that the matrix PGs are not derived from the PGs present in the medium and that an interaction between polysaccharide chains and matrix components is not sufficient for incorporation of PGs into the matrix. Incubation of cell-free ³⁵S-labelled matrix with unlabelled polysaccharides did not lead to the release of any ³⁵S-labelled material, supporting this conclusion. Furthermore, so-called 'link proteins' were not present in the fibroblast cultures, indicating that the CSPGs were anchored in the matrix in a manner different from the link-stabilized association of CSPG with HA in chondrocyte matrix. The identification of a proteinase, secreted by fibroblasts in culture, that after activation with heparin has the ability to release ³⁵S-labelled PGs from the matrix may also indicate that the core proteins are important for the association of the PGs to the matrix.

INTRODUCTION

One expression of the phenotypic state of cells is the nature of the extracellular matrix that they synthesize. Thus chondrocytes (Dessau *et al.*, 1981), myocytes (Beach *et al.*, 1982), fibroblasts (Hedman *et al.*, 1979) and endothelial cells (Leivo *et al.*, 1982) grown *in vitro* produce extracellular matrices of different compositions. Conversely, extracellular-matrix components appear to influence the phenotype of cells (Sieber-Blum *et al.*, 1978; West *et al.*, 1979). In order to understand the interrelations between cell phenotype and the matrix surrounding the cells, more knowledge is needed about the molecular organization of the different types of matrices and their mechanism of formation.

In the fibroblast matrix the glycosaminoglycans heparan sulphate (HS), chondroitin sulphate (CS) and hyaluronic acid (HA) have been identified as well as the major protein components fibronectin and procollagens type I and III (Hedman *et al.*, 1978). By immunofluorescence techniques, procollagens type I and III, a heparan sulphate proteoglycan (HSPG) and a portion of the chondroitin sulphate proteoglycans (CSPGs) were found to co-distribute with fibronectin in a fibrillar

network (Vaheri *et al.*, 1978; Hedman *et al.*, 1982). Whereas fibronectin and collagen types I and III are relatively well characterized, previous studies have revealed a marked heterogeneity of fibroblast PGs, obtained from the growth medium and the cell layer (Cöster *et al.*, 1979; Vogel & Peterson, 1981; Lark & Culp, 1982). The heterogeneity may partly be due to the complexity of the compartments defined. Thus the cell layer contains the extracellular matrix, cell-surface PGs and intracellular material undergoing biosynthesis or degradation. The relation of the PGs in the medium to those present in the cell layer is unclear, although PGs in the medium often are assumed to represent components destined for matrix incorporation and/or degradation products from matrix turnover. Also, the glycosaminoglycans present in the 'substrate-attached material', which is associated with the dish after EDTA-induced detachment of the cells, have been studied. 'Substrate-attached material' consists of pinched-off 'footpads', containing components involved in cell adhesion (Lark & Culp, 1982). However, this fraction of the cell culture has also been shown to be polydisperse, containing both intra- and extra-cellular macromolecules with a portion of the glycosaminoglycans in the form of free chains (Murray

Abbreviations used: PG, proteoglycan; CS, chondroitin sulphate (in this paper chondroitin sulphate is not distinguished from dermatan sulphate, and CS refers to polysaccharides degradable with chondroitinase ABC, according to the nomenclature suggested by Höök *et al.*, 1984); HS, heparan sulphate; HA, hyaluronic acid.

|| To whom correspondence should be addressed.

& Culp, 1981; Lark & Culp, 1984a, b). Most of the extracellular matrix is detached from the culture dish along with the cells during the EDTA treatment (Murray & Culp, 1981).

Isolation of pericellular matrix by solubilization of cultured cells with ionic detergents appears to generate a more defined compartment. The isolated matrix retains its structural integrity, and is essentially devoid of intracellular components (Hedman *et al.*, 1978). In the present paper, the isolation and basic characterization of a CSPG and an HSPG of the deoxycholate-prepared matrix is described. The anchoring of the PGs in the matrix is discussed.

MATERIALS AND METHODS

Materials

$\text{Na}_2^{35}\text{SO}_4$ (carrier-free) and Na^{125}I (carrier-free) were purchased from The Radiochemical Centre. Papain (type III), guanidinium chloride, phenylmethanesulphonyl fluoride, pepstatin A, EDTA, dithiothreitol and 1,10-phenanthroline were obtained from Sigma Chemical Co., and *N*-ethylmaleimide and L-cysteine from Merck. Sepharose CL-2B and CL-4B and Sephadex G-25 and G-50 were obtained from Pharmacia, and CPG 2500 (controlled-pore glass for permeation chromatography) was from Electro-Nucleonics (Fairfield, NY, U.S.A.).

Heparin (stage 14) from pig intestinal mucosa was purified as described previously (Lindahl *et al.*, 1965). HS isolated from bovine liver (preparation I) and HS from human aorta (preparation II) have been described previously (Kjellén *et al.*, 1980). CS from bovine nasal septa and HA from rooster combs were kindly given by Dr. Å. Wasteson and Dr. T. C. Laurent (University of Uppsala, Uppsala, Sweden) respectively. The HA preparation contained exclusively high-molecular-mass material, which was eluted in the void volume after gel chromatography on Sepharose CL-2B. HA_{16} (oligosaccharides of HA, consisting of 16 monosaccharide units) was prepared from a partial digest of HA with testicular hyaluronidase as described previously (Christner *et al.*, 1977). CSPG from human articular cartilage (A1D1) was kindly given by Dr. B. Caterson (School of Medicine, Morgantown, WV, U.S.A.). ^3H -labelled heparin (10000 c.p.m./ μg of hexuronic acid), CS (60000 c.p.m./ μg of hexuronic acid) and HA (70000 c.p.m./ μg of hexuronic acid) were prepared by partial *N*-deacetylation (hydrazinolysis) followed by re-*N*-acetylation with ^3H -acetic anhydride as described (Höök *et al.*, 1982).

Cell culture

Human embryonic fibroblasts were cultured in Eagle's Basal Medium supplemented with 10% (v/v) foetal-calf serum (Hedman *et al.*, 1978). Three days after subculture the cells were metabolically labelled with ^{35}S sulphate (0.1 mCi/ml) for 3 days (unless otherwise indicated). Extracellular matrix was isolated after solubilization of the cells by using the deoxycholate procedure (Hedman *et al.*, 1979). Conditioned medium of cells grown for 3 days was clarified by centrifugation at 3000 *g* for 10 min before use.

Isolation of PGs

^{35}S -labelled matrices were incubated with extraction buffer (containing 4 M-guanidinium chloride, 0.2%

Fig. 1. Gel chromatography on Sepharose CL-2B of macromolecules extracted from ^{35}S -labelled matrix (a) ^{35}S -labelled components with density ≥ 1.50 g/ml (b) and with density 1.34–1.38 g/ml (c) after CsCl-density-gradient centrifugation under dissociative conditions

The column (1 cm × 80 cm) was eluted at 4 °C with 0.02 M-Tris/HCl buffer, pH 8.0, containing 4 M-guanidinium chloride (extraction buffer) at a flow rate of 6 ml/h, and 1.9 ml fractions were collected and analysed for radioactivity. In this and subsequent Figures V_0 and V_t represent void volume and total volume respectively.

Triton X-100, 0.2 mM-phenylmethanesulphonyl fluoride, 1 mM-EDTA, 0.2% NaN_3 and 0.02 M-Tris/HCl buffer, pH 8.0) for 30 min at 4 °C and scraped off the dishes. The extract was subjected to density-gradient centrifugation in CsCl as described previously (Hedman *et al.*, 1982) with the modification that extraction buffer was used in the centrifugation. After dialysis and papain digestion of the PGs the glycosaminoglycans were identified by degradation with chondroitinase ABC (Sigma Chemical Co.) and HNO_2 as described previously (Hedman *et al.*, 1982). Alkali treatment of PGs was performed by incubating ^{35}S -labelled PGs in 0.5 M-NaOH at 22 °C for 72 h. The samples were neutralized with HCl before analysis.

Labelling with ^{125}I

^{125}I -labelling of CSPGs from fibroblast matrix and human articular cartilage respectively was performed by using Iodo-beads (Pierce Chemical Co., Rockford, IL, U.S.A.). CSPGs together with 10 μg of HA in 0.2 ml of 0.2 M-sodium phosphate buffer, pH 7.5, were labelled with 0.5 mCi of Na^{125}I . The iodinated PGs were isolated by chromatography of a Sephadex G-25 column (15 mm × 50 mm) equilibrated and eluted with 0.02 M-Tris/HCl buffer, pH 8.0, containing 0.5 M-NaCl, 0.2% Triton X-100, 0.2 mM-phenylmethanesulphonyl fluoride

Fig. 2. Gel chromatography on Sepharose CL-4B of ^{35}S -labelled polysaccharide chains obtained after alkali treatment of CsCl-density-gradient-purified CSPS (buoyant density ≥ 1.50 g/ml) (a) and HSPG (buoyant density 1.34–1.38 g/ml) (b)

The column (1 cm $\times$ 90 cm) was eluted in extraction buffer under the same conditions as indicated in Fig. 1 legend.

and 1 mM-EDTA and further purified by chromatography on Sepharose CL-2B eluted with extraction buffer.

Electrophoresis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed on polyacrylamide gradient gels (5–10%) with a 4% spacer gel (Blobel & Dobberstein, 1975). Human plasma fibronectin and reference proteins from Pharmacia were used as molecular-mass markers. After electrophoresis the fixed gel was dried and subjected to autoradiography with Kodak X-Omat AR films.

Radioactivity determinations

^{125}I radioactivity was measured in a Packard model 5260 Auto Gamma spectrometer, and ^{35}S radioactivity in a Packard model 2450 liquid-scintillation counter.

Immunochemical methods

Antisera raised against the HA-binding region of CSPG (Christner *et al.*, 1983) and link protein (Caterson *et al.*, 1979) from bovine nasal cartilage have been described previously. Immunoprecipitation was performed by incubating radioactively labelled PG in 0.1 ml of antiserum, diluted 1:5 in 0.02 M-Tris/HCl buffer, pH 8.0, containing 0.5 M-NaCl, 0.2% Triton X-100, 0.2% bovine serum albumin, 0.2 mM-phenylmethanesulphonyl fluoride, 1 mM-EDTA and 0.2% NaN_3 at 4 °C overnight. Protein A–Sepharose (30 μl of a 50%, v/v, suspension) was added and the incubation was continued for 1 h further at 22 °C. The radioactivity precipitated after washing three times with the incubation buffer was determined. Immunofluorescent staining, with anti-(link protein) serum, of cell layer and isolated matrix of fibroblasts and of chick chondrocyte cell layer was carried out on paraformaldehyde-fixed samples, with or without previous treatment with chondroitinase ABC, as described previously (Hedman *et al.*, 1982).

Fig. 3. Gel chromatography on Sepharose CL-2B of CSPG (a) and HSPG (b) preincubated in 0.5 ml for 30 min at 4 °C with (●) or without (○) 50 μg of HA

The column (1 cm $\times$ 80 cm) was eluted at 4 °C with 0.02 M-Tris/HCl buffer, pH 8.0, containing 0.5 M-NaCl, 0.2% Triton X-100, 0.2 mM-phenylmethanesulphonyl fluoride, 1 mM-EDTA, 0.01 M-benzamidine, 0.2% bovine serum albumin and 0.2% NaN_3 . The flow rate was 6 ml/h, and 2 ml fractions were collected and analysed for radioactivity.

RESULTS

Isolation and purification of ^{35}S -labelled matrix PGs

Human embryonic fibroblasts were cultured in the presence of [^{35}S]sulphate, and cell-free matrix was prepared by solubilization of the cells with deoxycholate. The matrix was scraped off the dishes after the addition of extraction buffer. More than 85% of the ^{35}S -labelled macromolecules were solubilized from the matrix, as determined by measuring ^{35}S radioactivity in a sample before and after centrifugation of the extract at 20000 g for 30 min. When chromatographed on a column of Sepharose CL-2B, eluted in the extraction buffer, the solubilized ^{35}S -labelled macromolecules were distributed in a broad peak (Fig. 1a) that on an average contained 63% HS and 37% CS. This composition was essentially constant from one preparation to another, and was identical with that recorded after papain digestion of the matrix without prior solubilization (results not shown).

The solubilized matrix components were subjected to CsCl-density-gradient centrifugation in the presence of guanidinium chloride. As previously shown (Hedman *et al.*, 1982), the ^{35}S -labelled macromolecules of the matrix could be separated into three pools. ^{35}S -labelled material recovered at densities ≥ 1.50 g/ml (approx. 25% of the total radioactivity recovered) was exclusively CS, whereas a peak of ^{35}S radioactivity with a buoyant density of 1.36 g/ml (approx. 65% of the total radioactivity recovered) contained 80% HS. ^{35}S -labelled macromolecules recovered at the top of the gradient (buoyant density ≤ 1.28 g/ml, approx. 10% of the total radioactivity) were mainly CS with small amounts of HS. The high-density CS fraction and the HS-rich peak were

Fig. 4. Gel chromatography on Sepharose CL-2B of culture medium obtained from fibroblasts grown in the presence of [³⁵S]sulphate (○), and of the same culture medium preincubated with 50 µg of HA/ml (●)

The labelled medium was dialysed against 0.14 M-NaCl/0.01 M-sodium phosphate buffer, pH 7.4, in order to remove inorganic [³⁵S]sulphate before the chromatography. The column (1 cm × 80 cm) was eluted under the same conditions and with the same buffer as indicated in Fig. 3 legend.

Fig. 5. Gel chromatography on CPG 2500 of ³⁵S-labelled PGS of the medium recovered in the void volume on Sepharose CL-2B without addition of HA

The pooled PGS in the peak were preincubated with (●) or without (○) 0.1 mg of HA₁₆/ml for 3 h at 22 °C before gel chromatography. The exclusion properties of the CPG 2500 gel are similar to those of Sepharose CL-2B. The column (0.5 cm × 45 cm) was eluted at 22 °C in the same buffer as indicated in Fig. 3 legend. The flow rate was 10 ml/h, and 0.5 ml fractions were collected and analysed for radioactivity.

further analysed. Gel chromatography on Sepharose CL-2B in the presence of 4 M guanidinium chloride showed K_{av} values of 0.35 and 0.38 respectively for the CS and HS fractions (Figs. 1b and 1c). The PG nature of the two fractions was evident from their susceptibility to alkali treatment. Polysaccharide chains from either fraction, liberated from the core proteins by this treatment, were eluted with a peak K_{av} value of 0.50 (Fig. 2) off a column of Sepharose CL-4B. The intact PGs were eluted in the void volume of this column (results not shown). On Sephadex G-200 both CS and HS chains were eluted close to the void volume, ahead of the CS

chains of known molecular mass (Wasteson, 1971) used for calibration of the column (results not shown). On the basis of these results the molecular mass of the CS chains and HS chains was estimated to be higher than 40 kDa.

Interaction of PGs with HA

PGs from cartilage, and also from some other tissues, are known to bind HA (Hascall & Heinegård, 1974; Norling *et al.*, 1978; Cöster *et al.*, 1979; Anderson, 1982; Wight & Hascall, 1983; Schafer *et al.*, 1984). An interaction between matrix PGs and HA could be partially responsible for the anchorage of the PGs in the fibroblast matrix. In order to investigate this possibility the purified matrix PGs, obtained after CsCl-density-gradient centrifugation, were incubated with an excess of HA under associative conditions. Analysis of the product by gel chromatography on Sepharose CL-2B showed that approx. 50% of the CSPG changed elution position and was excluded from the gel (Fig. 3). Thus at least a portion of the matrix CSPGs has the ability to bind HA. In contrast, the elution pattern for the matrix HSPG was not affected by incubation of the PG with HA (Fig. 3).

An HA-binding CSPG was present also in the culture medium. When ³⁵S-labelled PGs of the medium were chromatographed under the same conditions as above, without preincubation with HA, a small portion of the ³⁵S radioactivity was recovered in the void volume (Fig. 4), possibly representing aggregates of PGs and endogenous HA. Chondroitinase ABC digestion indicated that CS represented 91% of these excluded ³⁵S-labelled macromolecules, but only 55% of the unfractionated medium PS. When the PGs of the medium had been preincubated with HA, a small but significant increase in the relative amount of ³⁵S-labelled macromolecules eluted in the void volume was observed (Fig. 4). When the endogenous ³⁵S-labelled PG aggregates were incubated with HA oligosaccharides (HA₁₆) and rechromatographed on a similar column, more than 80% of previously excluded ³⁵S-labelled macromolecules were eluted as partially included material (Fig. 5), indicating that at least a majority of the aggregates were not stabilized by link proteins, in contrast with CSPG-HA aggregates in chondrocyte cultures (Kimura *et al.*, 1980). The lack of immunofluorescent staining of the fibroblast matrix, with anti-(link protein) antibodies that strongly stained the matrix of chondrocyte cultures (results not shown) furthermore suggested that link proteins are not involved in anchoring of CSPGs in the fibroblast matrix.

Comparison of CSPGs obtained from fibroblast matrix and cartilage

The molecular mass of the protein core of the CSPG purified from fibroblast matrix was determined and related to that of the aggregating PG from articular cartilage. For this purpose the PGs were labelled with ¹²⁵I and subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis before and after removal of the polysaccharide chains by chondroitinase ABC digestion. [The preparation of cartilage CSPGs contained differently sized PGs, as evident from sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 6). This heterogeneity in size has previously been observed for PGs isolated from whole tissues (Oegema *et al.*, 1977) and probably reflects the turnover of PGs in the tissue. In addition, some low-molecular-mass components, which migrate in the gel front, are present in the preparation.]

Fig. 6. Autoradiography after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of ^{125}I -labelled CSPGs

^{125}I -labelled PGs were dialysed against 0.1 M-Tris/HCl buffer, pH 8.0, containing 0.2 mM-phenylmethanesulphonyl fluoride, 1 mM-EDTA and 0.8 mM-*N*-ethylmaleimide. Pepstatin was added to a concentration of 10 $\mu\text{g}/\text{ml}$, and 0.1 ml of the ^{125}I -labelled PGs was incubated with 0.1 unit of chondroitinase ABC at 37 °C for 1 h. Samples containing 50000 c.p.m., before and after digestion with chondroitinase ABC, were reduced and applied to a gradient polyacrylamide gel. Electrophoresis was performed as described in the Materials and methods section and followed by autoradiography. Lane 1, cartilage CSPG; lane 2, chondroitinase ABC-digested cartilage CSPG; lane 3, fibroblast matrix CSPG; lane 4, chondroitinase ABC-digested matrix CSPG.

The core protein of the fibroblast PG migrated at a somewhat lower rate than did the cartilage PG (Fig. 6). Apparent molecular masses of 290 kDa and 250 kDa were obtained for the two core proteins respectively. The two HA-binding CSPGs were also immunologically compared by subjecting ^{35}S -labelled and ^{125}I -labelled fibroblast CSPG and ^{125}I -labelled cartilage CSPG to immunoprecipitation by using an antiserum raised against the HA-binding region of cartilage CSPG. Whereas no significant amount of the fibroblast CSPG was precipitated (0.5% and 2.5% of total ^{35}S radioactivity

and total ^{125}I radioactivity respectively, compared with 0.5% and 1.3% for non-immune serum), ^{125}I -labelled CSPG from cartilage was precipitated to an appreciable extent (23% compared with 0.4% for non-immune serum).

Effect of exogenous glycosaminoglycans on PG-matrix association

To investigate the role of the polysaccharide chains of the PGs and that of HA in the association of the PGs with the matrix, fibroblasts were grown in [^{35}S]sulphate-containing medium in the presence of exogenous glycosaminoglycans. High concentrations of HA₁₆ in the medium caused a decrease of macromolecular ^{35}S radioactivity to the same extent both in the matrix and in the medium (Table 1). Thus the presence of HA₁₆ seemed to affect the biosynthesis and/or secretion of PGs rather than matrix assembly. In contrast, addition of heparin to the culture medium decreased the amount of ^{35}S radioactivity incorporated into the matrix and increased the amount of macromolecular ^{35}S radioactivity present in the medium (Table 1), whereas HS and CS had little or no effect. At a heparin concentration of 20 $\mu\text{g}/\text{ml}$ the incorporation of ^{35}S radioactivity into the matrix was inhibited by 70%. The total amounts of HS or CS synthesized by the cells were not markedly affected by the addition of any of the exogenous sulphated polysaccharides.

These results could indicate that added heparin has the ability to compete with endogenous PGs for binding to the matrix. However, no binding to the matrix could be demonstrated when ^3H -labelled heparin or other glycosaminoglycans were included in the culture medium over the cells (Table 2). Moreover, when cell-free [^{35}S]sulphate-labelled matrix was isolated and incubated with heparin no significant amount of ^{35}S radioactivity was released (Table 3). Nor did incubations with HS, HA or HA₁₆ in concentrations up to 1 mg/ml affect the amount of ^{35}S radioactivity associated with the matrix (results not shown).

A possible explanation for the decreased amount of ^{35}S -labelled macromolecules incorporated into the matrix in the presence of heparin was provided by experiments in which heparin along with conditioned medium was added to isolated ^{35}S -labelled matrix. More than 40% of the matrix-associated radioactivity was released after 1 h of incubation (Table 3) although the matrix fibres remained bound to the dishes. Conditioned medium alone or heparin in physiological buffer had no such effect (Table 3). Furthermore, the heparin-dependent release of ^{35}S radioactivity in the presence of conditioned medium could be partly inhibited by pepstatin A or 1,10-phenanthroline, inhibitors of carboxy- and metallo-endopeptidases respectively (Barrett, 1977). The addition of phenylmethanesulphonyl fluoride (inhibitor of serine proteinases) or EDTA in combination with dithiothreitol [known to stimulate cysteine proteinases (Barrett, 1977)], did not significantly affect the amount of ^{35}S radioactivity released.

The PGs present in the medium were of smaller size than the matrix PGs (Figs. 1 and 4). In order to investigate the relationship between the PGs in the two compartments, ^{35}S -labelled PGs of the medium were incubated with unlabelled cells. The PGs of the medium failed to become incorporated into the matrix (Table 2), indicating that they are unrelated to or degradation

Table 1. [³⁵S]Sulphate incorporation into macromolecules synthesized by cells grown in the presence of exogenous glycosaminoglycans

Cells grown for 3 days in 35 mm dishes were incubated for 21 h at 37 °C in medium containing 0.1 mCi of [³⁵S]sulphate/ml and the indicated amounts of exogenously added glycosaminoglycans. The ³⁵S-labelled HS content of the matrix and medium was determined as described in the Materials and methods section. The amounts of radioactivity recovered in polysaccharides in matrix and in matrix + medium and that recovered in HS in matrix + medium in cultures without added glycosaminoglycans were set to 100.

Addition		³⁵ S-labelled polysaccharide in matrix		³⁵ S-labelled polysaccharide in matrix + medium		³⁵ S-labelled HS in matrix + medium	
		(10 ⁻³ × c.p.m.)	(%)	(10 ⁻³ × c.p.m.)	(%)	(10 ⁻³ × c.p.m.)	(%)
Expt. 1	None	421	100	1323	100	739	100
	1 mg of HA ₁₆ /ml	316	75	994	75	510	69
	1 mg of HA ₁₆ + 1 mg of CS/ml	249	59	1030	78	522	71
	20 µg of heparin/ml	124	29	1366	103	775	105
	60 µg of heparin/ml	33	8	1210	92	730	100
	100 µg of heparin/ml	16	4	1197	91	686	93
Expt. 2	None	121	100	569	100		
	100 µg of heparin/ml	17	14	575	101		
	100 µg of HS (I)/ml	86	71	555	98		
	100 µg of HS (II)/ml	120	99	562	99		
	100 µg of CS/ml	110	91	516	91		
	1000 µg of CS/ml	104	86	569	100		

Table 2. Distribution of exogenously added glycosaminoglycans in fibroblast cultures

[³⁵S]Sulphate-labelled growth medium, dialysed to remove inorganic [³⁵S]sulphate, and ³H-labelled glycosaminoglycans were added to fibroblasts grown in 35 mm dishes for 3 days. After 24 h the medium was collected and the cell pool was recovered after solubilization in deoxycholate. The matrix was dissolved by incubation in 1 M-NaOH for 1 h at 22 °C. Medium, cell pool and matrix pool were analysed for radioactivity.

Addition	Radioactivity recovered (c.p.m.)		
	In medium	In cell pool	In matrix pool
³ H-labelled heparin	22100	612	29
³ H-labelled CS	33000	197	17
³ H-labelled HA	3100	42	14
³⁵ S-labelled macromolecules of medium	4600	134	51

Table 3. Release of [³⁵S]Sulphate-labelled components from cell-free matrices

[³⁵S]Sulphate-labelled matrices (approx. 80000 c.p.m./dish), isolated from cells grown in 35 mm dishes, were incubated at 37 °C in 1 ml of the indicated solutions. The buffer contained 137 mM-NaCl, 4.7 mM-KCl, 0.6 mM-MgSO₄ and 1.2 mM-CaCl₂ in 10 mM-Hepes/NaOH buffer, pH 7.4. After 1 h the supernatants were collected and the matrices were scraped off the dishes in 1 ml of extraction buffer. ³⁵S radioactivities of both fractions were then determined.

Matrix incubated with	³⁵ S radioactivity released (%)
Buffer	4.3
Buffer + 0.1 mg of heparin/ml	6.6
Conditioned medium	1.7
Conditioned medium + 0.1 mg of heparin/ml	43.9
Conditioned medium + 0.1 mg of heparin/ml + phenylmethanesulphonyl fluoride (1 mM)	41.6
Conditioned medium + 0.1 mg of heparin/ml + EDTA (2 mM) + dithiothreitol (2 mM)	37.8
Conditioned medium + 0.1 mg of heparin/ml + pepstatin A (10 µg/ml)	22.1
Conditioned medium + 0.1 mg of heparin/ml + 1,10-phenanthroline (2 mM)	21.0

products from the matrix PGs, rather than precursors to the matrix PGs.

DISCUSSION

The interactions between PGs and other macromolecules in the extracellular space are generally assumed to contribute to the functional properties of the tissues. The aim of the present study has been to characterize the PGs of a defined extracellular matrix, and to elucidate the mode of binding between PGs and other matrix macromolecules.

The CSPG of fibroblast matrix showed affinity for HA, a property characteristic of cartilage PGs, but later also demonstrated for PGs from other sources. In spite of this functional similarity, some structural differences between cartilage and fibroblast CSPGs could be observed: (1) the CS chains from the fibroblast PG had a higher molecular mass than had the PS of the cartilage CSPG [> 40 kDa as compared with 20 kDa (Oegema *et al.*, 1977)]; (2) the apparent molecular mass of the core protein obtained from the fibroblast matrix CSPG was somewhat higher than that of the cartilage PG; (3) cartilage CSPG, but not fibroblast CSPG, was precipitated by antibodies prepared against the HA-binding region of the cartilage PG, in agreement with a previous report (Vertel & Dorfman, 1979). Whether the observed differences reflect variations in the post-translational modification reactions of a common protein core or the expression of different genes cannot be ascertained from any of these data.

As previously reported (Cöster *et al.*, 1979), an HA-binding CSPG was also present in the medium of the fibroblast cultures. In the present study it was shown that most of the aggregates from the medium could be dissolved by the addition of HA₁₆, indicating that the PGs had been associated with endogenous HA, and that no link proteins were present. In contrast, the corresponding aggregates formed in chondrocyte cultures are stabilized by link proteins (Kimura *et al.*, 1980). Immunofluorescence staining, with anti-(link protein) antibodies, strongly stained the matrix of chondrocyte cultures but failed to stain the fibroblast matrix, further suggesting that fibroblasts in culture do not synthesize link proteins. Interestingly, no link proteins could be detected in cultures of de-differentiated chondrocytes (Oegema & Thompson, 1981a, b), although these cells synthesized an HA-binding PG.

The fibroblast matrix HSPG had a similar size to that of the HSPG isolated from matrix produced by glial cells (Norling *et al.*, 1981) and cannot be distinguished from the HSPG extracted from fibroblast cell layers (Vogel & Peterson, 1981; Carlstedt *et al.*, 1983). The cell-layer HSPG has been estimated to have a molecular mass of approx. 350 kDa (Carlstedt *et al.*, 1983).

PGs from the fibroblast culture medium did not bind to the matrix when given to fibroblast cultures. This is in contrast with fibronectin, which is secreted into the medium and becomes incorporated into matrix fibres after binding to a 'matrix-assembly receptor' on the cell surface (Oh *et al.*, 1981; McKeown-Longo & Mosher, 1983, 1985). Both HSPGs (Kjellén *et al.*, 1981; Norling *et al.*, 1981) and CSPGs (Oldberg *et al.*, 1981; Hedman *et al.*, 1983) have been shown to occur as membrane-associated forms in fibroblasts and other cell types. Thus the membrane-bound PGs may be the precursors of the

matrix PGs and complex with fibronectin on the cell surface.

Neither the HSPG nor the CSPG could be displaced from isolated matrix by various exogenous polysaccharides at high concentrations, indicating that the core proteins are involved in anchoring the PGs to the matrix. Since HA₁₆ did dissolve aggregates of CSPG present in the culture medium but was ineffective in releasing any PG from the matrix, additional interactions besides binding to HA apparently operate to bind CSPG to the matrix. The finding that conditioned medium in combination with heparin resulted in a release of radioactivity from ³⁵S-labelled matrix suggests that (a) proteolytic enzyme(s) in the medium were activated by heparin. Inhibition of the releasing activity by proteinase inhibitors pointed to one or both of two classes of enzymes, carboxy- and metallo-endopeptidases. The mechanism of heparin activation in the fibroblast system is as yet unknown and will require further investigation.

This work was supported by grants from the Swedish Medical Research Council (6525 and 13X-07147).

REFERENCES

- Anderson, J. C. (1982) *Biochem. Biophys. Res. Commun.* **107**, 1390–1394
- Barrett, A. J. (1977) in *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., ed.), pp. 1–55, Elsevier/North-Holland Biomedical Press, Amsterdam
- Beach, R. L., Burton, W. V., Hendricks, W. J. & Festoff, B. W. (1982) *J. Biol. Chem.* **257**, 11437–11442
- Blobel, G. & Dobberstein, B. (1975) *J. Cell Biol.* **67**, 835–851
- Carlstedt, I., Cöster, L., Malmström, A. & Fransson, L.-Å. (1983) *J. Biol. Chem.* **258**, 11629–11635
- Caterson, B., Baker, J. R., Levitt, D. & Pasley, J. W. (1979) *J. Biol. Chem.* **254**, 9369–9372
- Christner, J. E., Brown, M. C. & Dziewiatkowski, D. D. (1977) *Biochem. J.* **167**, 711–716
- Christner, J. E., Baker, J. R. & Caterson, B. (1983) *J. Biol. Chem.* **258**, 14335–14341
- Cöster, L., Carlstedt, I. & Malmström, A. (1979) *Biochem. J.* **183**, 669–681
- Dessau, W., Vertel, B. M., von der Mark, H. & von der Mark, K. (1981) *J. Cell Biol.* **96**, 78–83
- Hascall, V. C. & Heinegård, D. (1974) *J. Biol. Chem.* **249**, 4242–4249
- Hedman, K., Vaheri, A. & Wartiovaara, J. (1978) *J. Cell Biol.* **76**, 748–760
- Hedman, K., Kurkinen, K., Alitalo, K., Vaheri, A., Johansson, S. & Höök, M. (1979) *J. Cell Biol.* **81**, 83–91
- Hedman, K., Johansson, S., Vartio, T., Kjellén, L., Vaheri, A. & Höök, M. (1982) *Cell (Cambridge, Mass.)* **28**, 663–671
- Hedman, K., Christner, J., Julkunen, I. & Vaheri, A. (1983) *J. Cell Biol.* **97**, 1288–1293
- Höök, M., Risenfeld, J. & Lindahl, U. (1982) *Anal. Biochem.* **119**, 235–245
- Höök, M., Kjellén, L., Johansson, S. & Robinson, J. (1984) *Annu. Rev. Biochem.* **53**, 847–869
- Kimura, J. H., Hardingham, T. E. & Hascall, V. C. (1980) *J. Biol. Chem.* **255**, 7134–7143
- Kjellén, L., Oldberg, Å. & Höök, M. (1980) *J. Biol. Chem.* **255**, 10407–10413
- Kjellén, L., Pettersson, I. & Höök, M. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 5371–5375
- Lark, M. W. & Culp, L. A. (1982) *J. Biol. Chem.* **257**, 14073–14080
- Lark, M. W. & Culp, L. A. (1984a) *J. Biol. Chem.* **259**, 212–217
- Lark, M. A. & Culp, L. A. (1984b) *J. Biol. Chem.* **259**, 6773–6782

- Leivo, I., Alitalo, K., Vaheri, A., Timpl, R. & Wartiovaara, J. (1982) *Exp. Cell Res.* **137**, 15–23
- Lindahl, U., Cifonelli, L. A., Lindahl, B. & Rodén, L. (1965) *J. Biol. Chem.* **240**, 2817–2820
- McKeown-Longo, P. J. & Mosher, D. F. (1983) *J. Cell Biol.* **97**, 466–472
- McKeown-Longo, P. J. & Mosher, D. F. (1985) *J. Cell Biol.* **100**, 364–374
- Murray, B. A. & Culp, L. A. (1981) *Exp. Cell Res.* **131**, 237–249
- Norling, B., Glimelius, B., Westermark, B. & Wasteson, Å. (1978) *Biochem. Biophys. Res. Commun.* **84**, 914–921
- Norling, B., Glimelius, B. & Wasteson, Å. (1981) *Biochem. Biophys. Res. Commun.* **103**, 1265–1272
- Oegema, T. R. & Thompson, R. C. (1981a) *J. Biol. Chem.* **256**, 1015–1022
- Oegema, T. R. & Thompson, R. C. (1981b) *J. Biol. Chem.* **256**, 4494–4498
- Oegema, T. R., Brown, M. & Dziewiatkowski, D. D. (1977) *J. Biol. Chem.* **252**, 6470–6477
- Oh, E., Piersbacher, M. & Ruoslahti, E. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3218–3221
- Oldberg, Å., Hayman, E. G. & Ruoslahti, E. (1981) *J. Biol. Chem.* **256**, 10847–10852
- Schafer, I. A., Sitabkha, L. & Pandey, M. (1984) *J. Biol. Chem.* **259**, 2321–2330
- Sieber-Blum, M., Sieber, F. & Yamada, K. M. (1978) *Exp. Cell Res.* **133**, 285–292
- Vaheri, A., Kurkinen, M., Lehto, V.-P., Linder, E. & Timpl, R. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4944–4948
- Vertel, B. & Dorfman, A. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1261–1264
- Vogel, K. G. & Peterson, D. W. (1981) *J. Biol. Chem.* **256**, 13235–13242
- Wasteson, Å. (1971) *Biochem. J.* **122**, 477–485
- West, C. M., Langa, R., Rosenbloom, J., Lowe, M., Holtzen, H. & Avdalovic, H. (1979) *Cell (Cambridge, Mass.)* **17**, 491–501
- Wight, T. N. & Hascall, V. C. (1983) *J. Cell Biol.* **96**, 167–176

Received 4 March 1985/17 June 1985; accepted 15 July 1985