Heparin proteoglycans synthesized by mouse mastocytoma contain chondroitin sulphate

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Proteoglycans (PGs), biosynthetically labelled with [³⁵S]sulphate, were isolated from mouse mastocytoma tissue. Chromatography on antithrombin (AT)–Sephacel resulted in the separation of the ³⁵S-labelled PGs into three fractions: PGs with no affinity for the gel (NA-PGs), PGs with low affinity (LA-PGs), and PGs with high affinity (HA-PGs) for antithrombin. Whereas NA-PGs contained almost exclusively chondroitin sulphate (CS), the AT-binding PGs contained 80–85% heparin and 15–20% CS. [³⁵S]CS-containing macromolecules obtained from the HA-PG fraction after removal of the heparin polysaccharide chains were rechromatographed on AT–Sephacel. A majority of these ³⁵S-labelled macromolecules no longer showed affinity for AT. These experiments indicate that the [³⁵S]CS recovered in the AT-binding PGs is present in hybrid PGs. Polysaccharide chain-

length determination demonstrated that the heparin chains were somewhat larger ($M_r \sim 30000$) than the CS chains in the NA-PGs ($M_r \sim 25000$). CS chains in the hybrid PGs were slightly smaller ($M_r \sim 20000$). Characterization of the sulphated CS disaccharides from NA- and HA-PGs showed that they contained similar amounts (20%) of disulphated disaccharides of [GlcA-GalNAc(4,6-di-OSO₃)] type. The monosulphated CS-disaccharides were O-sulphated at C-4 of the galactosamine units. Analysis by gel chromatography of the [³⁵S]CS components isolated from HA-PGs after heparinase treatment showed that a major portion of these contained one CS chain only. Calculations of the number of CS and heparin chains in AT-binding PGs, based on polysaccharide composition and polysaccharide chain length, indicate that all heparin-containing PGs are hybrids.

INTRODUCTION

During the last years, the primary structure of several proteoglycan (PG) core proteins has been determined (for a review see [1]). However, it is still not possible to judge from the amino acid sequence if a certain protein will be selected as a PG core protein. Both chondroitin sulphate (CS) and heparan sulphate (HS)/heparin chains are attached to serine residues, which are often, but not always, followed by a glycine residue. What then determines if the glycosaminoglycan chain will be of the CS or HS/heparin type? Most PG core proteins carry either CS or HS, and many cell types at the same time synthesize several PGs with different glycosaminoglycan chains. Apparently, some mechanism selects a certain protein for HS substitution, while another protein will be present in a CSPG. However, some PGs are hybrids and carry CS and HS chains on the same core protein. The most studied hybrid PGs, syndecans [2,3], have been shown to contain different amounts of CS and HS in different cell types [4]. Studies of PGs with potential glycosaminoglycan attachment sites spread along the core protein have indicated that structural elements of the core protein determine if CS or HS will be added to a particular site [5,6].

During mast-cell differentiation the glycosaminoglycan synthesis shifts from CS, present in mucosal mast cells, to heparin, found in connective-tissue-type mast cells. It has been shown that the same core protein, serglycin, is present in both kinds of PGs [7]. This protein was first identified in a rat yolk-sac carcinoma where it carries CS chains [8], but has later been found to be present in different haemopoietic cells (for a review see [9]). The protein is unusual in that it contains a region of serine and glycine in repeating sequence. For serglycin, it has not been

established whether the same serine residue can be substituted with either CS or HS, or if the two types of glycosaminoglycan chains have different, distinct, sites of attachment in the Ser-Gly repeat. However, for heparin PGs isolated from rat skin, it has been shown that two-thirds of the serine residues are substituted with polysaccharide [10]. If the CS-containing serglycins are also as heavily substituted, a certain serine residue in this core protein must be able to accommodate either CS or heparin. In line with this notion, it has been suggested that at least some of the PGs in RBL-1 cells, later shown to contain serglycin [11], are hybrids containing both CS and heparin chains [12].

In the present paper we have characterized the PGs synthesized by a mouse mastocytoma. The results presented indicate that approximately half of the serglycins in these cells are substituted with CS only, whereas the other half are present in hybrid PGs of a defined composition, each containing several heparin chains and one CS chain. No 'pure' heparin-PG seems to be synthesized by the cells.

MATERIALS AND METHODS

A transplantable mouse mastocytoma, originally described by Furth et al. [13], was maintained in the laboratory by routine intramuscular passage every 10–12 days in the hind legs of (A/Sn × Leaden)F₁ mice.

DEAE-Sephacel, Sephacryl S400 HR, Sepharose CL-6B, Sephadex G-50, Sephadex G-15 and the Superose 6 column were obtained from Pharmacia. Antithrombin (AT)–Sephacel [14] was kindly given by Dr. Ulf Lindahl, Uppsala, Sweden. Carrier-free [³⁵S]sulphate was obtained from New England Nuclear. Heparinase and chondroitinase ABC were purchased from

Abbreviations used: aMan₆, 2,5-anhydro- α -mannitol; AT, antithrombin; CS, chondroitin sulphate; GalNAc, *N*-acetyl- α -galactosamine; GlcA, α -glucuronic acid; HA-PG, proteoglycan with high affinity for antithrombin; HexA, unspecified hexuronic acid; HS, heparan sulphate; IdoA, *L*-iduronic acid; LA-PG, proteoglycan with low affinity for antithrombin; NA-PG, proteoglycan with no affinity for antithrombin; PG, proteoglycan.

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Seikagaku, Japan. Standards for paper electrophoresis were kindly given by Dr. Ulf Lindahl, Uppsala, Sweden, and consisted of the monosulphated disaccharide GlcA- ^3H aMan $_6$ (6-OSO $_3$), the monosulphated monosaccharide ^3H aMan $_6$ (6-OSO $_3$) and the disulphated disaccharide IdoA(2-OSO $_3$)- ^3H aMan $_6$ (6-OSO $_3$) (where aMan $_6$ is 2,5-anhydro-D-mannitol and IdoA is L-iduronic acid). Unsaturated monosulphated-disaccharide standards for paper chromatography, D-HexA-GalN ^3H Ac(6-OSO $_3$) and D-HexA-GalN ^3H Ac(4-OSO $_3$) (where HexA is an unspecified hexuronic acid and GalNAc is *N*-acetyl-D-galactosamine), were kindly given by Dr. Å. Wasteson, Linköping, Sweden. The hyaluronan fragment standards of known molecular size have previously been described [15].

Purification of mastocytoma [^{35}S]PGs

Two tumour-bearing mice were injected (intraperitoneally) with 2 mCi of [^{35}S]sulphate/animal. After 2 h the tumours were dissected out and homogenized in 25 ml of 1% Triton X-100/0.05 M Tris/HCl, pH 8.0, containing the protease inhibitors PMSF (1 mM), *N*-ethylmaleimide (2 mM), EDTA (2 mM) and pepstatin (10 $\mu\text{g}/\text{ml}$). These protease inhibitors, at the indicated concentrations, were present in all subsequent buffers used for the purification of the [^{35}S]PGs. After an end-over-end incubation at 4 °C for 30 min, the homogenate was centrifuged for 1 h at 100 000 *g* and the supernatant was used for purification of the [^{35}S]PGs as previously described [7], the procedure involving DEAE-Sephacel ion-exchange chromatography and gel filtration on Sephacryl S400 HR.

Specific degradation of PGs and polysaccharide chains

Deamination with nitrous acid at pH 1.5 was performed by the method of Shively and Conrad [16] (which degrades heparin to short oligosaccharides) followed by gel chromatography on Sephadex G-50 to separate CS, which is eluted in the void volume, from heparin degradation products.

Heparinase and chondroitinase ABC digestions of isolated ^{35}S -proteoglycans were carried out as previously described [7].

Alkali treatment was performed in 0.5 M NaOH at 4 °C for 20 h.

Affinity chromatography on AT-Sephrose

Preparative and analytical affinity chromatography was performed on a 3 ml column of AT-Sephrose connected to a LKB HPLC gradient mixer, ensuring reproducible elution. After application of the sample in 0.05 M Tris/HCl, pH 7.4, containing 0.05 M NaCl, the column was washed with 24 ml of the same buffer and subsequently eluted with a linear salt gradient (120 ml) ranging from 0.05 M to 3.0 M NaCl in 0.05 M Tris/HCl, pH 7.4. The column was eluted at 0.2 ml/min and 2 ml fractions were collected.

Analytical gel chromatography

Chromatography on Sepharose CL-6B was performed on a 1 cm $\times$ 90 cm column eluted at 6.6 ml/h in 0.05 M Tris/HCl, pH 8.0, containing 0.15 M NaCl and 0.1% SDS. Fractions of volume 2.2 ml were collected and analysed for radioactivity.

HPLC on Superose 6 was performed on a 1 cm $\times$ 30 cm column eluted at 0.5 ml/min in 0.01 M Tris/HCl, pH 7.4, containing 1 M NaCl. Fractions of volume 1 ml were collected and analysed for radioactivity.

Isolation and characterization of CS disaccharides

CS disaccharides, generated after chondroitinase ABC digestion of ^{35}S -labelled PGs, were isolated by chromatography on Sephadex G-15 (1 cm $\times$ 170 cm) equilibrated with 0.2 M NH_4HCO_3 . The disaccharides were freeze-dried and dissolved in water before analyses.

The glycosidic linkage between the unsaturated hexuronic acid residue and the galactosamine residue was cleaved by treatment with 10 mM mercuric acetate at room temperature for 30 min [17].

High-voltage electrophoresis was conducted on Whatman 3MM paper in 1.6 M formic acid (pH 1.7; 40 V/cm). Paper chromatography was performed on similar paper and developed with acetic acid/*n*-butanol/1 M NH_4OH (3:2:1, by vol.) for 29 h. After drying, papers were cut into 1 cm segments, which were transferred to vials for liquid-scintillation counting of radioactivity. The paper segments were extracted with 1 ml of water for 1 h before addition of 5 ml of scintillation cocktail.

RESULTS

PGs biosynthetically labelled with [^{35}S]sulphate were isolated from mouse mastocytoma tissue as previously described [7]. After alkali treatment, the polysaccharide composition of the released glycosaminoglycan chains was determined by gel chromatography on Sephadex G-50 after deamination with nitrous acid (which results in depolymerization of heparin) and after treatment with chondroitinase ABC (which degrades CS). The heparin content of different preparations was shown to range between 40 and 60% (results not shown). Gel chromatography on a Superose 6 column showed that heparin [^{35}S]PGs were larger (after removal of the CS with chondroitinase ABC) than the [^{35}S]CSPG (recovered after HNO_2 treatment) (Figure 1). Also the ^{35}S -labelled heparin chains had a higher apparent molecular mass than the [^{35}S]CS chains (Figure 1). Relating the elution positions to those of hyaluronan standards, molecular masses of $\sim 30\,000$ and $\sim 25\,000$ respectively could be estimated for the heparin and CS chains.

Chromatography of PGs on AT-Sephrose

Since heparin is known to bind to AT [18], affinity chromatography on AT-Sephrose was used to separate the heparin- and

Figure 1 Gel chromatography on Superose 6 of mastocytoma heparin and CS PGs and their respective polysaccharide chains

Purified ^{35}S -labelled mastocytoma PGs were treated with chondroitinase ABC or nitrous acid, and the resulting resistant ^{35}S -heparin ($\circ$, $\bullet$) and [^{35}S]CS containing ($\square$, $\blacksquare$) macromolecules were isolated and chromatographed on a Superose 6 column, as described in the Materials and methods section, before ($\circ$, $\square$) and after ($\bullet$, $\blacksquare$) alkali treatment. The arrows indicate, from left to right, the elution positions of defined hyaluronan fragments (M_r 43 000, M_r 30 000, M_r 19 000).

Figure 2 Preparative affinity chromatography on AT-Sepharose of ^{35}S -labelled mastocytoma PGs

Mastocytoma PGs corresponding to 1.5×10^6 c.p.m. of ^{35}S were chromatographed on AT-Sepharose as described in the Materials and methods section. A 20 μl sample of each fraction was analysed for ^{35}S radioactivity. Fractions containing NA-, LA- and HA-PGs were pooled as indicated in the Figure.

Table 1 Heparin content of NA, LA and HA pools obtained after chromatography on AT-Sepharose of ^{35}S -PGs and alkali-released ^{35}S -labelled polysaccharides

AT fraction	^{35}S -PGs		^{35}S -labelled polysaccharides	
	^{35}S radioactivity recovered, c.p.m. (% of total)	Heparin content* (%)	^{35}S radioactivity recovered, c.p.m. (% of total)	Heparin content* (%)
NA	740 700 (58)	6	254 100 (69)	7
LA	344 500 (27)	82	74 000 (20)	100
HA	191 000 (15)	86	40 400 (11)	100

* Heparin content was determined after alkali treatment and nitrous acid deamination followed by gel chromatography on Sephadex G-50. The numbers in parentheses indicate the percentage of the total ^{35}S radioactivity recovered in the different fractions.

CS-containing ^{35}S -PGs. As shown in Figure 2, the PGs were divided into three different populations after chromatography on AT-Sepharose. A large portion of the PGs did not bind to the gel ('no affinity' = NA-PGs), whereas after gradient elution of the column, PGs with low affinity for AT (LA-PGs) were eluted early in the gradient, and PGs with high affinity (HA-PGs), requiring a higher salt concentration for elution, were recovered. Polysaccharide analyses of the three fractions, pooled as indicated in Figure 2, showed that NA-PGs contained almost exclusively CS, whereas LA-PGs and HA-PGs consisted of 80–85 % heparin (Table 1).

When alkali-released ^{35}S -glycosaminoglycan chains from the same PG preparation were similarly chromatographed on AT-Sepharose, no CS could be detected in the AT-binding fractions (Table 1). This result indicates that the CS recovered in the AT-binding fractions of the ^{35}S -PG preparation was present in hybrid PGs. Alternatively, due to the polyvalent nature of the CSPGs, these could have a greater affinity for the affinity matrix than single CS chains.

To test the hybrid hypothesis, the heparin chains were removed from the AT-binding PGs by nitrous acid treatment, and the affinity of the remaining CS macromolecules for AT was investigated. (As is evident from Figure 1, nitrous acid treatment does not depolymerize CSPGs to free polysaccharide chains.) Rechromatography of the HA-PGs on AT-Sepharose before

Figure 3 Analytical rechromatography on AT-Sepharose of intact ^{35}S -labelled HA-PGs and ^{35}S -labelled HA-PGs devoid of heparin chains

^{35}S -HA-PGs obtained as described in the legend to Figure 2 were chromatographed on AT-Sepharose before (a) and after removal of the heparin chains by nitrous acid degradation (b).

Figure 4 Gel chromatography on Sepharose CL-6B of ^{35}S -labelled HA-PGs devoid of heparin chains

[^{35}S]CS-containing macromolecules obtained after heparinase digestion of HA-PGs were chromatographed on Sepharose CL-6B before (○) and after (●) alkali treatment.

nitrous acid treatment resulted in elution of the PGs at the same position as previously (compare Figures 2 and 3a). In contrast, after removal of the heparin polysaccharide chains, a majority of the nitrous acid-resistant [^{35}S]CS-containing macromolecules were no longer able to bind to the column, and a small proportion of the ^{35}S -labelled macromolecules were eluted in the position of LA-PGs (Figure 3b). Similar results were obtained when LA-PGs were treated with nitrous acid, reisolated and rechromatographed on the AT-Sepharose column (results not shown). These results strongly indicate that CS eluted in the AT-binding fraction is present in hybrid PGs. Interestingly, a majority of these PGs seemed to contain one CS chain only, since most of the ^{35}S -labelled CS components obtained after heparinase digestion did not change their elution position on Sepharose CL-6B after alkali treatment (Figure 4).

Figure 5 Gel chromatography on Superose 6 of ^{35}S -labelled polysaccharide chains obtained from HA- and NA-PGs

^{35}S -labelled heparin chains (●) obtained from HA-PGs after alkali treatment of chondroitinase-ABC-resistant ^{35}S -labelled macromolecules, and ^{35}S -labelled CS chains obtained from HA-PGs (▲) and NA-PGs (■) after alkali treatment of nitrous acid-resistant ^{35}S -labelled macromolecules, were chromatographed on Superose 6 as described in the Materials and methods section. The size markers are the same as those used in the gel chromatography shown in Figure 1.

Characterization of the CS chains from NA- and HA-PGs

When CS chains obtained from NA-PGs and HA-PGs were analysed by gel chromatography, it could be shown that the CS polysaccharide chains present in the HA-PGs were somewhat smaller ($M_r \sim 20000$) than CS chains obtained from NA-PGs ($M_r \sim 25000$; Figure 5). Hence, in the HA-PGs, the heparin chains are about 1.5 times larger than the chondroitin chains.

To investigate whether also the structure of the CS chains in the hybrids differed from that of the CS in the NA-PG fraction, [^{35}S]CS disaccharides (recovered after chondroitinase digestion) from the two fractions were analysed by paper electrophoresis at pH 1.7. As shown in Figure 6, both preparations had a similar content of monosulphated (65% of the total ^{35}S radioactivity) and disulphated disaccharides (35% of the total ^{35}S radioactivity). Since the ^{35}S radioactivity is twice as high in the disulphated as in the monosulphated disaccharides, the actual proportion of disulphated disaccharides is approx. 20%, whereas the rest of the sulphated disaccharides carry one sulphate group/disaccharide. The overall content of sulphate groups in the CS chain would thus be $\sim 1.2/\text{disaccharide}$.

Treatment of the disaccharides from NA-PGs and HA-PGs with mercuric acetate, which cleaves the glycosidic bond between the monosaccharide units, resulted in the generation of two types of ^{35}S -labelled monosaccharides that could be separated by electrophoreses (Figure 6). The largest peak, containing 65% of the total ^{35}S radioactivity in both the NA-PG and HA-PG preparations, migrated slightly more slowly than the monosulphated-monosaccharide standard, whereas the remaining 35% of the ^{35}S radioactivity was found in a peak with a higher mobility than the disulphated heparin disaccharide standard (Figure 6). Monosulphated and disulphated monosaccharides obtained from CS have previously been shown to have these migration properties [19]. The relative amount of ^{35}S radioactivity in the disulphated-monosaccharide peak (35%) is identical with that obtained for the disulphated disaccharide before mercuric acetate treatment (Figure 6), indicating that all disulphated disaccharides were composed of one non-sulphated and one disulphated monosaccharide. That this actually was the case was shown for the mono- and di-sulphated disaccharides obtained from NA-PGs, which were isolated after preparative electrophoresis. When the ^{35}S -labelled disulphated disaccharides

Figure 6 Paper electrophoresis of unsaturated ^{35}S -labelled disaccharides generated by digestion of CS with chondroitinase ABC, before and after treatment with mercuric acetate

Unsaturated disaccharides derived from NA-PGs (a) and HA-PGs (b) were subjected to paper electrophoresis at pH 1.7 as described in the Materials and methods section before (●) and after treatment with mercuric acetate (○). The arrows indicate the positions of ^3H -labelled standards: the monosulphated disaccharide GMS, $\text{GlcA}-[^3\text{H}]\text{aMan}_6(6\text{-OSO}_3)$; the monosulphated monosaccharide MS, $[^3\text{H}]\text{aMan}_6(6\text{-OSO}_3)$; the disulphated disaccharide ISMS, $\text{IdoA}(2\text{-OSO}_3)-[^3\text{H}]\text{aMan}_6(6\text{-OSO}_3)$.

were separately cleaved with mercuric acetate and analysed by paper electrophoresis, all ^{35}S radioactivity was present in the disulphated monosaccharide fraction, whereas the monosulphated monosaccharides were exclusively derived from the monosulphated disaccharides (results not shown). Paper chromatography of the monosulphated disaccharide fraction

Figure 7 Paper chromatography of unsaturated monosulphated disaccharides obtained from NA-PGs after chondroitinase ABC digestion

Monosulphated ^{35}S -labelled NA-PG disaccharides obtained after preparative paper electrophoresis at pH 1.7, as shown in Figure 6, were subjected to paper chromatography as described in the Materials and methods section. The arrows indicate the positions of the unsaturated monosulphated-disaccharide standards: $\Delta\text{Di-6S}$, $\Delta\text{HexA-GalN}([^3\text{H}]\text{Ac}(6\text{-OSO}_3)$; $\Delta\text{Di-4S}$, $\Delta\text{HexA-GalN}([^3\text{H}]\text{Ac}(4\text{-OSO}_3)$.

(isolated after preparative paper electrophoresis) from the NA-PGs showed that they co-migrated with the 4-*O*-sulphated standard disaccharide (Figure 7). Taken together, these results show that the CS chains of the hybrid and NA-PGs both contain 20% HexAGalNAc(4,6-di-OSO₃) disaccharides, whereas the rest of the sulphated disaccharides, at least in the NA-PGs, have the structure HexAGalNAc(4-OSO₃).

DISCUSSION

The mouse mastocytoma used in the present study has been extensively studied with regard to the mechanisms of heparin biosynthesis (see [20] and references therein). However, CS is also produced by the tumour, and we have previously shown that both types of glycosaminoglycans are attached to the same core protein, serglycin [7]. The present study indicates that a substantial portion of these PGs are hybrids, containing both CS and heparin.

CS chains, liberated from the PGs by alkali treatment, did not bind to AT-Sephrose (Table 1). However, CS was present in the AT-binding fractions when intact PGs were chromatographed on AT-Sephrose (Table 1). Removal of the heparin chains from the AT-binding PGs by nitrous acid treatment prevented the majority of the remaining CS macromolecules from binding to the column (Figure 3). These results strongly suggest that the CS found in the AT-binding fractions is present in hybrid PGs. The possibility that CSPGs were non-covalently associated with the AT-binding heparin PGs seems unlikely, since heparinase digestion of the HA-PGs resulted in the generation not of CSPGs but of CS macromolecules containing mostly one CS chain (Figure 5).

Structural analyses of the [³⁵S]CS disaccharides showed that the sulphate groups were present on the galactosamine units exclusively, 20% being 4,6-disulphated (referred to as CS-E) [21], whereas the rest contained one sulphate group at position C-4 (CS-A). Comparing this structure with that found for CS isolated from rat mucosal mast cells [19], both preparations appear to contain similar amounts of monosulphated disaccharides, which are of the CS-A type also in the mucosal mast cells. However, although the disulphated disaccharides in the mastocytoma CS were exclusively of the CS-E type, the mucosal mast cells in addition contain CS-B, with the general structure -IdoA(2-OSO₃)-GalNAc(4-OSO₃)-.

Assuming that all disaccharides in mastocytoma CS are sulphated, the sulphate content would be 1.2/disaccharide. This is considerably less than that of the mastocytoma heparin, which is 2.5 sulphate groups/disaccharide (K. Lidholt, I. Eriksson and L. Kjellén, unpublished work). Furthermore, the heparin polysaccharide chains seemed to be 1.5 times as large as the CS chains present in the hybrid PGs (Figure 5). Taken together, the larger amount of [³⁵S]-sulphate groups/disaccharide and the larger size of the heparin chains suggest that a heparin chain contains 3 times the amount of ³⁵S radioactivity as that found in a CS chain of a hybrid PG. Ten serine residues which could carry glycosaminoglycan chains are present in the Ser-Gly repeat region of mouse serglycin [7]. If it is assumed that (1) the ³⁵S content is 3 times higher in a heparin chain than in a CS chain, due to longer chains and a higher degree of sulphation, and (2) 86% of the ³⁵S radioactivity is present in heparin (as in the HA-PGs; Table 1), the average number of CS chains per PG would be ≥ 1 as long as the PG contains at least three polysaccharide chains. Considering that most of the hybrid PGs seemed to contain just one CS chain (Figure 4), it is apparent that very few, if any, PGs carry heparin only.

The presence of the two defined types of mastocytoma PGs, the NA-PG substituted with CS chain only and the hybrid heparin PGs containing one additional CS chain, indicates a certain specificity in the substitution of the polysaccharide chains. However, since the core proteins are identical, structural elements in the core protein cannot be responsible for the difference in glycosaminoglycan content of the hybrid and the CSPG. Different modification of the linkage region Galβ1 → 3Galβ1 → 3Xylβ → Ser, present in both CS and heparin chains, could be one means of regulating the biosynthesis. Although phosphate groups at C-2 of the xylose residue have been detected in both kinds of polysaccharides [22–24], only the linkage region of CS has been shown to contain sulphate groups attached to the galactose residues [25–29].

Since the mastocytoma, as well as the RBL-1 (rat basophilic leucocyte 1) cell line suggested to synthesize serglycin hybrids [12], are tumour cells, the production of hybrids may be a transformation-related phenomenon. It will therefore be important to investigate whether PGs synthesized by normal mast cells are also hybrids. Chromatography on AT-Sephrose should be a useful method for this purpose, since small amounts of cells that can be labelled with [³⁵S]sulphate *in vitro* would be sufficient to produce the quantities of ³⁵S-PGs needed for the analyses.

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