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Biochemical characterization of integral membrane heparan sulfate proteoglycans in Sertoli cells from immature rat testis

Sylvie Brucato *, Gaëlle Fagnen, Corinne Villers, Pierre-Jacques Bonnamy, Monique Langris, Jean Bocquet

Laboratoire de Biochimie, UPRES EA 2608, I.R.B.A., Université de Caen, Esplanade de la Paix, F-14032 Caen Cedex, France

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Abstract

^{35}S-Radiolabeled cultured Sertoli cells from immature rat testis were extracted with detergent and the different proteoheparan sulfate (HSPG) forms of the extract were discriminated and quantified on the basis of their high anionic charge, hydrodynamic size, lipophilic properties, susceptibility to trypsin and phosphatidylinositol phospholipase C (PI-PLC). Trypsin released 50% of total cellular HSPG corresponding to 80% of total hydrophobic HSPG. Trypsin-accessible HSPG were presumed to be integral membrane species. Trypsin-resistant HSPG, probably intracellular, distributed into non-lipophilic (37.5%) and lipophilic (12.5%) populations. Biochemical analysis of PG copurified with plasma membrane confirmed the existence of hydrophobic HSPG integrated into this structure. Among hydrophobic HSPG accessible to trypsin, 35% were PI-PLC released and radiolabeled by [^{3}H]inositol indicating that about one third of integral membrane HSPG were intercalated into the plasma membrane through a phosphatidylinositol anchor (glypican type). PI-PLC-resistant forms represented HSPG inserted into the membrane through a hydrophobic segment of the core protein (syndecan type). No lipophilic PG was present in other cell compartments (culture medium, cell periphery, extracellular matrix). ^{125}I-Iodinated hydrophobic HSPG were deglycanated and submitted to SDS-polyacrylamide gel electrophoresis. In the glypican family, a core protein (64–65 kDa) was detected, whereas in the syndecan family, bands of 60 and 68 kDa were observed which may correspond to self-association of different core proteins. In Sertoli cell, specific functional attributes of different integral membrane HSPG forms remain to be investigated.

Keywords: Membrane heparan sulfate proteoglycan; Sertoli cell; Rat

1. Introduction

Heparan sulfate proteoglycans (HSPG) are composed of a core protein to which negatively charged and fine structure heparan sulfate (HS) chains are covalently attached [1,2]. In animal tissues, HSPG are mostly located in two major areas: in the extracellular matrix, and particularly in the basement membrane where they take part in the development of supramolecular architecture, and at the cell surface. Basement membrane is a specialized sheet-like extracellular matrix, biochemically complex. Its heterogeneous structure consists in laminin, collagen type IV, nidogen and HSPG such as perlecan, agrin [3] and, recently identified, collagen type XVIII [4].

Cell surface HSPG are associated to the plasma membrane according to three modes. The first con-

* Corresponding author. Fax: +33-2-31-56-23-20; E-mail: biochimie@ibba.unicaen.fr

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sists in a direct intercalation of the core protein into the lipid bilayer, through a transmembrane hydrophobic sequence (transmembrane HSPG). The second is realized by a linkage to the external leaflet of the membrane through a glycosylphosphatidylinositol (GPI) moiety covalently bound to the core protein (GPI-HSPG). Transmembrane HSPG, which notably include syndecan-1, -2, -3, -4 [5], betaglycan (type III transforming growth factor β (TGFβ) receptors) [6,7] and splice variants of CD44 (hyaluronan receptors) [8,9], and GPI-HSPG, which are mostly represented by the glypican family (glypican-1 to -6) [10,11], are both designated as integral membrane HSPG. Syndecans and glypicans are individually expressed in distinct cell-, tissue- and development-specific patterns [5,10]. The third mode of cell surface association concerns the peripheral HSPG which achieved interactions of their HS chains with sites referred to membrane receptors [12]. Relatively high concentrations of free heparin or salt solutions displace these peripheral HSPG from their receptors but do not release integral membrane HSPG which are efficiently extracted with detergent [13].

Cell surface-associated HSPG play highly diversified roles relative to their localization, to the ability of HS chains, and less extensively, of core proteins [14] to interact with numerous molecules. An increasing variety of HS ligands have been described, including extracellular matrix proteins, cell-cell adhesion molecules, proteolytic and lipolytic enzymes, lipoproteins, protease inhibitors, growth factors, cytokines, chemokines, microorganisms such as viruses, bacteria and protozoa [5]. Thus, cell surface HSPG may participate in cell adhesion, proliferation and motility, bioavailability regulation and activity of soluble polypeptide factors, modulation of lipid metabolism, attachment of pathogenic agents and invasion of host cells.

In seminiferous tubules of testis, epithelial type Sertoli cells provide structural support for the development of germinal cells and are in contact by their basal surface with a basement membrane [15]. Basement membrane is cooperatively produced by Sertoli and peritubular cells which surround the seminiferous tubules [16], these two cell types synthesizing HSPG [17,18]. In addition to the synthesis of extracellular HSPG, Sertoli cells elaborated HSPG with hydrophobic features suggesting an integral membrane disposition [19].

Sertoli cells are the target of many growth factors, for example, basic fibroblast growth factor (bFGF) and TGFβ which regulates estradiol production [20]. Although not evidenced in Sertoli cells, it is very likely that, as in other cells, integral membrane HSPG take part in the activation of bFGF [21,22] and TGFβ [23] signaling receptors.

Moreover, we have shown that synthesis inhibition [24] and sulfation degree lowering [25] of Sertoli cell proteoglycans are accompanied by an increase in follicle stimulating hormone (FSH)-stimulated estradiol synthesis. Modifications of estradiol production resulted from a decrease in cyclic adenosine monophosphate (cAMP) phosphodiesterase activity and a steroidogenesis stimulation in a post-cAMP step [24,25]. These data indicated that proteoglycans (PG) functionally interfere with FSH signal transduction.

Thus, due to their strategic position between extracellular and intracellular compartments, integral membrane HSPG may constitute good candidates for this regulation, but they are not yet characterized in Sertoli cells. As a first biochemical approach, the present work aims at clarifying the cell distribution of Sertoli cell HSPG and studying their attachment mode to the plasma membrane. Biochemical analysis indicated the existence of both transmembrane HSPG and GPI-HSPG.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's minimal essential medium (DMEM), Ham's F-12 medium, sulfate-free and sulfate/inositol-free DMEM/Ham's F-12 medium were from J. Boy (Reims, France). Bovine pancreas deoxyribonuclease (DNase type I), hyaluronidase (type I-S), Triton X-100, *n*-octyl-D-glucopyranoside, L-phosphatidylcholine (type V-E), protease inhibitors, test kits for 5′-nucleotidase and for acid phosphatase were purchased from Sigma (Saint Quentin Fallavier, France). Phosphatidylinositol phospholipase C (PI-PLC from *Bacillus cereus*) and collagenase-dispase were obtained from Boehringer

Mannheim (Meylan, France). Trypsin was from Gibco-BRL (Cergy Pontoise, France). Ultroser SF (steroid-free serum substitute) and DEAE-Trisacryl were provided by IBF-Biotechnics (Villeneuve la Garenne, France). Sepharose CL-2B, Sepharose CL-4B, octyl-Sepharose CL-4 B and FPLC Mono Q column were from Pharmacia (Saint Quentin-Yvelines, France). Heparitinase (EC 4.2.2.8; heparitin sulfate lyase) was from Miles Laboratories (Coger, Paris, France). [^{35}S]Sulfate (39–59 Tbq/mg) and [^{125}I]Na (629 GBq/mg) were from Amersham (Les Ulis, France). *myo*-[2-^{3}H(N)]Inositol (2.96–4.44 TBq/mmol) was obtained from NEN (Les Ulis, France). Cationic nylon (Zeta-Probe) was purchased from Bio-Rad (Ivry sur Seine, France). All other reagents were of analytical biology grade.

2.2. Preparation of Sertoli cell cultures

Twenty-day-old Sprague-Dawley rats from our own colony were killed by cervical dislocation. Testicular Sertoli cells were obtained by sequential enzymatic digestion including trypsin, collagenase, hyaluronidase and deoxyribonuclease, as previously described [26,27]. Cells were plated at a concentration of 2.5×10^5 cells/cm^2 in 75 cm^2 plastic flasks and cultured for 24 h in Ham's F-12-DMEM containing 2% Ultroser SF, in a humidified atmosphere of 95% air/5% CO_2, at 32°C. After 48 h, culture medium was discarded and replaced by free-Ultroser SF Ham's F-12-DMEM medium. On day 3 of culture, residual germinal cells were removed by brief hypotonic treatment using 20 mM Tris-HCl (pH 7.4) [27]. Sertoli cells were used on day 5 after plating.

2.3. Radiolabeling

Confluent Sertoli cells were metabolically radiolabeled for 48 h in sulfate-free Ham's F-12-DMEM containing 5 μCi/ml of carrier-free sulfate [^{35}S]sulfate or, in some experiments, by [^{35}S]sulfate (5 μCi/ml) and [^{3}H]inositol (5 μCi/ml) in sulfate- and inositol-free medium.

2.4. Isolation of plasma membrane

A plasma membrane-enriched fraction of Sertoli cells was isolated as essentially described by Lories et al. [28]. Cell cultures, labeled by [^{35}S]sulfate, were washed and scraped in 1 mM $NaHCO_3$ with a rubber policeman, at 4°C. The cell suspension, adjusted to 1 mM EDTA, was homogenized with a Potter-Elvehjem homogenizer and centrifugated ($1000\times g$, 10 min). After centrifugation of the preceding supernatant ($25\,000\times g$, 30 min), crude membrane preparation was recuperated. This fraction was resuspended in 10% (w/w) sucrose solution in PBS, layered on top of a 32% (w/w) sucrose solution in PBS and centrifugated ($100\,000\times g$, 2 h). The majority of the plasma membrane, recovered at the interface of the sucrose solutions, was washed with PBS, heparin solution (100 mg/ml in PBS), and solubilized in 4 M guanidine chloride in 10 mM Tris-HCl buffer, pH 7.2, in the presence of protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 10 mM *N*-ethylmaleimide, 100 mM 6-aminohexanoic acid, 1 g/ml pepstatin, 20 mM EDTA and 5 mM benzamidine-HCl).

Activities of enzymatic subcellular markers (5′-nucleotidase and acid phosphatase for plasma membrane and lysosomes respectively) were assessed, according to the manufacturer's instructions, in supernatant of crude $NaHCO_3$ homogenate and in plasma membrane-enriched fraction. Protein concentrations were determined by the Bradford method [29].

2.5. Analysis of PG

2.5.1. Extraction procedure

After labeling with the different precursors (see Section 2), culture medium was collected, the cell layer was washed twice with PBS and washings were added to the medium. After addition of protease inhibitors at the required concentrations, the sample was centrifugated ($1000\times g$, 15 min). Then, the cell layer was treated twice with PBS containing 100 mg/ml heparin and protease inhibitors, for 30 min at 4°C. Except for media used during enzymatic digestion (see below), all buffers needed in the subsequent extractions, purifications and analyses contained the same mixture of protease inhibitors.

After elimination of peripheral PG by heparin, the cell layer was either treated with trypsin (100 mg/ml in PBS) for 3 min at 37°C and the digestion was stopped by soybean trypsin inhibitor (50 mg/ml), or

with PI-PLC (0.2 IU/ml in Ham's F-12-DMEM) for 1 h at 37°C. Material spontaneously secreted (control secretion) during enzymatic treatments was determined by incubating Sertoli cells for 3 or 60 min in trypsin- and PI-PLC-free medium respectively. PG radioactivity was determined in incubation medium after enzymatic treatment and, at the same time, in medium control, by solid phase assay (see Section 2.5.2.). The amount of enzymatically released PG was calculated by subtracting the radioactivity of control cell medium from the radioactivity of trypsin- or PI-PLC-treated cell medium.

Cell layer, treated or not with hydrolytic enzymes, was extracted with 0.5% (v/v) Triton X-100, 10 mM Tris-HCl (pH 7.2) for 15 min at 37°C, under gentle shaking. The detergent extract was cleared by centrifugation ($10\,000 \times g$, 30 min).

Finally, cell layer not submitted to digestion by trypsin or PI-PLC, but sequentially treated with heparin and Triton X-100, was extracted with Tris-HCl buffer (10 mM, pH 7.2) in the presence of 8 M urea and 0.5% Triton X-100 (24 h at 4°C). PG of this extract were referred to extracellular matrix-associated PG.

2.5.2. Quantitative assay for HSPG and HS chains

PG and glycosaminoglycan (GAG) quantification was performed by solid phase assay with slight modifications [30]. Samples were adjusted to 4 M urea, 0.5% Triton X-100, 50 mM Tris-HCl, 0.15 M NaCl, pH 7.0 and spotted on four different cationic nylon membranes, previously soaked in 50 mM Tris-HCl buffer, pH 7.0 (TBS) containing 0.15 M NaCl, and sandwiched in a dot blot apparatus. Under these conditions, non-anionic and weakly anionic molecules did not bind to the membrane. Two of the four membranes were washed twice for 30 min on a shaker platform, in TBS containing 0.9 M NaCl to remove free GAG chains and their degradation products and bind only PG. In addition, one 0.15 M NaCl-treated blot and one 0.9 M NaCl-treated blot were submitted to nitrous acid treatment to specifically assess HSPG- and HS-associated radioactivity [31]. After rinsing with distilled water and ethanol, blots were cut in segments bearing the imprint of an individual dot and submitted to liquid scintillation counting. PG radioactivity was directly determined on 0.9 M NaCl-treated dot. GAG radioactivity was calculated by subtracting radioactivity of 0.9 M NaCl-treated dot from that of 0.15 M NaCl-treated dot.

Results of quantitative analysis were the means of four different experiments (Table 1).

2.5.3. Ion-exchange chromatography

Cell culture extracts were dialyzed against 10 mM Tris-HCl (pH 7.2) buffer containing 4 M urea and 0.5% Triton X-100, and purified by passage over a DEAE-Trisacryl column (5 ml) previously equilibrated with the same buffer. After sample application, the column was rinsed with 10 ml of equilibration buffer and then eluted with a gradient of NaCl

Table 1
Forms and distribution of [^{35}S]heparan sulfate from Triton X-100 extract of cultured Sertoli cells[a]

	% of total ^{35}S incorporated into all HS forms	% of total ^{35}S HSPG	% of total ^{35}S hydrophobic HSPG
HS-free chains and their degradation products	68 ± 10.2	–	–
Non-hydrophobic HSPG (intracellular presumed)	12 ± 2.2	37.5 ± 5.6	–
Hydrophobic HSPG resistant to trypsin	4 ± 1.0	12.5 ± 3.4	20 ± 3.7
Hydrophobic HSPG accessible to trypsin (integral membrane HSPG) and distributed among	16 ± 1.9	50 ± 4.2	80 ± 8.9
PI-PLC susceptible HSPG	5 ± 0.6	16 ± 1.7	25.5 ± 2.7 (32%)[b]
PI-PLC resistant HSPG	11 ± 0.9	34 ± 4.1	54.5 ± 4.9 (68%)[b]

[a]HS-free chains and HSPG were quantified after anion-exchange chromatography, hydrophobic interaction chromatography and solid phase assay on cationic nylon blot (see Section 2). These results are representative of four experiments (mean ± S.E.).
[b]Values in parentheses are calculated as percentage of total integral membrane HSPG (transmembrane HSPG+GPI-HSPG).

(0–1.0 M in a total volume of 50 ml). A flow rate of 25 ml/h was used and 1 ml fractions were collected. Alternately, elution was performed stepwise, with 0.2 and 1.0 M NaCl, in urea buffer (each 20 ml).

PG fractions resulting from ion-exchange chromatography were pooled and concentrated. After adjusting their NaCl concentration to 0.15 M by adding 4 M urea buffer, they were loaded onto a 0.2 ml DEAE-Trisacryl column. Bound PG were displaced by a small volume (2 ml) of 4 M guanidine-HCl in 10 mM Tris-HCl, pH 7.2, 0.5% Triton X-100.

In some experiments, PG purification was performed by FPLC on a Mono Q column, bound material being eluted by a linear NaCl gradient (0–1.2 M) in Tris-HCl/urea buffer.

2.5.4. Gel chromatography

Plasma membrane extract was submitted to gel chromatography on Sepharose CL-2B and Sepharose CL-4B (1×55 cm) and elution was carried out with 4 M guanidine-HCl buffer, respectively in absence or presence of Triton X-100. Size exclusion of radiolabeled PG was performed on Sepharose CL-4B (1×55 cm) with 4 M urea buffer as eluent. Flow rate of each gel filtration was 6 ml/h and 0.4 ml fractions were collected.

2.5.5. Hydrophobic interaction chromatography and liposome intercalation

Triton X-100 present in the different purified PG extracts was exchanged for dialyzable octyl glucoside detergent. PG samples were dialyzed against 10 mM Tris-HCl buffer, 4 M urea, pH 7.2 and loaded onto a 0.5 ml DEAE-Trisacryl column equilibrated with the same buffer. The column was washed sequentially with 20 ml of Tris-HCl urea buffer and with 10 ml of Tris-HCl urea buffer containing 50 mM octyl glucoside. Bound proteoglycans were displaced with 10 mM Tris-HCl buffer, pH 7.2, 4 M guanidine chloride, 75 mM octyl glucoside. Samples were dialyzed against 10 mM Tris-HCl buffer, pH 7.2, 4 M guanidine chloride, then mixed with 1.5 ml of octyl-Sepharose CL-4B and put overnight on a shaker, at 4°C. Octyl-Sepharose CL-4B gel was transferred to a small column and the gel was successively eluted with 15 ml of 10 mM Tris-HCl buffer, pH 7.2, 4 M guanidine chloride, and 15 ml of the same buffer containing 1% Triton X-100. Fractions of 1 ml were collected.

To test PG insertion into liposomes, Triton X-100 present in the samples was exchanged for octyl glucoside by the method described above. Incorporation of DEAE-Trisacryl purified lipophilic PG into liposomes was performed in the presence of phosphatidylcholine. L-α-Phosphatidylcholine from egg yolk, solubilized in chloroform/methanol (9:1) was evaporated to dryness under a stream of nitrogen. Sample, dissolved in 10 mM Tris-HCl buffer, pH 7.2, 4 M guanidine chloride containing 75 mM octyl glucoside, was added to phosphatidylcholine (5 mg of phospholipid/ml of sample solution). The mixture was dialyzed overnight against 10 mM Tris-HCl buffer, pH 7.2, 4 M guanidine chloride without octyl glucoside, at room temperature, to induce intercalation of hydrophobic material into liposomes. PG insertion into phosphatidylcholine micelles was revealed by gel filtration on Sepharose CL-4B (1×55 cm), with detergent-free 10 mM Tris-HCl buffer, pH 7.2, 4 M guanidine chloride as eluent.

2.5.6. Enzymatic digestion and nitrous acid degradation

Purified HSPG were dissolved in a buffer pH 7.0 containing 50 mM Tris-HCl, 100 mM NaCl, 100 mg/ml bovine serum albumin, 50 mM 6-aminohexanoic acid, 2.5 mg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 20 mg/ml leupeptin. After addition of heparitinase (60 mIU/ml), the mixture was incubated for 3 h at 37°C. Alternately, heparan side chains were degraded by deaminative cleavage using the pH 1.5/HNO_2 method [31].

2.6. Core protein electrophoresis

Purified hydrophobic HSPG (extracted by Triton X-100 and successively submitted to ion-exchange chromatography and hydrophobic interaction chromatography) were treated by PI-PLC (0.6 U/ml) for 2 h at 37°C. After dialysis, the resulting material was mixed with phosphatidylcholine and size fractionation was performed on Sepharose CL-4B (see Section 2.5.5.). Proteoglycans intercalated and non-intercalated into lipidic vesicles corresponded respectively to transmembrane HSPG and GPI-HSPG which

have lost their hydrophobic properties after PI-PLC treatment.

The two HSPG families were dissolved in 100 mM Tris-HCl buffer, pH 7.2, containing 10 mM octyl glucoside and separately bound to DEAE-Trisacryl (1 ml) equilibrated in the same buffer. After 30 min at 4°C, the suspension was centrifugated ($3000 \times g$, 5 min), the supernatant discarded and the anion-exchange matrix was washed with Tris-HCl buffer. Membrane HSPG bound to DEAE-Trisacryl were iodinated with [^{125}I]Na (1 mCi) and chloramine T (1 mg/ml), for 5 min at room temperature, and the reaction was stopped by adding $K_2S_2O_5$ (5 mM) and KI (10 mM). DEAE-Trisacryl matrix was abundantly rinsed with 100 mM Tris-HCl (pH 7.2) buffer containing 4 M urea and 0.5% Triton X-100 to eliminate free [^{125}I]Na. [^{125}I]HSPG were eluted with the same buffer containing 1 M NaCl, purified by FPLC-Mono Q chromatography, dialyzed against distilled water and lyophilized. After heparitinase digestion, ^{125}I-labeled core proteins were submitted to electrophoresis.

Linear gradient gel (4–15% polyacrylamide) was used in a denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system, with the buffer system of Laemmli. Before electrophoresis, samples were adjusted at 2% (w/v) in electrophoresis buffer and boiled for 5 min. Samples running under reducing conditions were supplemented with 2% β-mercaptoethanol. After electrophoresis (6 h, 30 mA/gel), gel was fixed in 50% methanol-3% acetic acid, rinsed in distilled water, dried under vacuum, and subjected to autoradiography in the presence of Kodak XAR-5 film.

Results of qualitative analysis were representative of three different experiments.

3. Results

3.1. Cell surface-associated HSPG

In the preparation of Sertoli cell plasma membranes, obtained on sucrose discontinuous gradient, 5′-nucleotidase was increased 13.4-fold in specific activity relative to the initial homogenate (6404 ± 843 IU/mg protein vs. 476.8 ± 63.7 IU/mg protein, $n = 3$) and acid phosphatase was increased 3.4-fold

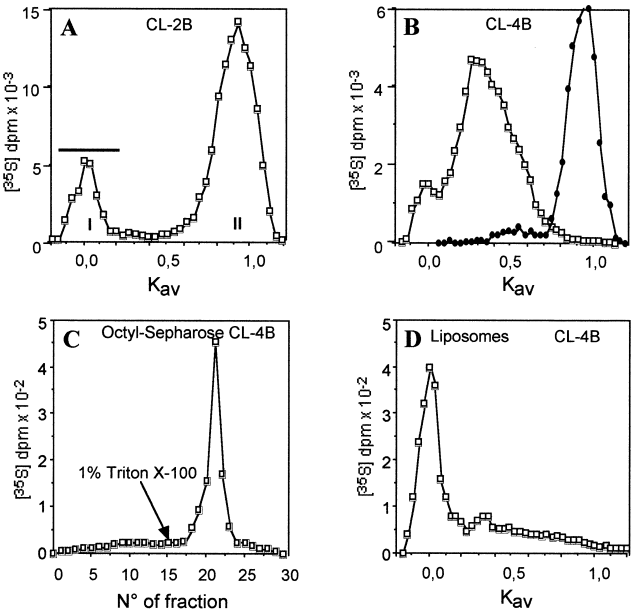

Fig. 1. Cell surface-associated HSPG. (A) Purified plasma membrane of [^{35}S]sulfate-labeled Sertoli cells was extracted with free detergent Tris-HCl buffered 4 M guanidine chloride and the extract was chromatographed on Sepharose CL-2B in the same buffer. (B) Peak I of panel A (bar indicated) was supplemented with Triton X-100 (5% final concentration) and chromatographed on Sepharose CL-4B in Tris-HCl buffered 4 M guanidine chloride containing 5% Triton X-100, before (□) and after (●) nitrous acid degradation. Hydrophobic features of HSPG from peak I were assessed, as described in Section 2, by (C) hydrophobic interaction chromatography on octyl-Sepharose CL-4B (hydrophobic HSPG were eluted by buffer containing 1% Triton X-100) and (D) by intercalation into phosphatidylcholine liposomes and gel filtration on Sepharose CL-4B.

(0.078 ± 0.009 IU/mg protein vs. 0.023 ± 0.005 IU/mg protein, $n = 3$). These results indicated that fraction recovered at the interface of sucrose solutions (10–32%) was mostly enriched in plasma membranes and partially contaminated by lysosomes.

The plasma membrane preparation was solubilized in 4 M guanidine chloride-Tris HCl buffer, at 4°C for 24 h and chromatographed on Sepharose CL-2B (Fig. 1A). ^{35}S-Radiolabeled material distributed as a peak in the void volume (peak I) and a peak at $K_{av} = 0.90$ (peak II). Fractions of peak I were pooled (bar indicated), concentrated, adjusted to 5% Triton X-100 final concentration, chromatographed on Sepharose CL-4B with guanidine chloride buffer containing 5% Triton X-100 as eluent (Fig. 1B). Under these conditions, about 90% of the ^{35}S-labeled material originally emerging in the void volume of the

Sepharose CL-2B, eluted as single peak at $K_{av} = 0.33$. This material was totally susceptible to nitrous acid degradation (Fig. 1B). These results showed that peak I contained exclusively HSPG copurifying with plasma membrane. When extracted from plasma membrane by detergent-free chaotropic solvent, HSPG interacted with membrane lipids to form aggregates which were disrupted by Triton X-100. Peak II on Sepharose CL-2B ($K_{av} = 0.90$) may represent PG degradation products, associated to contaminating lysosomal fraction, and previously detected in Sertoli cell Triton X-100 extract [19].

The lipophilic features of peak I HSPG were assessed by hydrophobic interaction chromatography and intercalation into liposomes. HSPG were essentially retained on octyl-Sepharose CL-4B (Fig. 1C) and eluted with 1% Triton X-100. Furthermore, HSPG were incorporated into phosphatidylcholine micelles since they were detected in the void volume of the Sepharose CL-4B column instead of emerging at $K_{av} = 0.33$ (Fig. 1D).

3.2. Cell layer trypsin-susceptible HSPG

Trypsin can release integral membrane PG. After removing the ^{35}S-metabolically labeled Sertoli cell culture medium, the cell layer was successively washed with PBS and heparin solution, treated or not by trypsin (100 mg/ml) and extracted by 0.5% Triton X-100. The detergent extracts from control and trypsin-treated cell layer were anion-exchange purified and chromatographed on Sepharose CL-4B (Fig. 2A).

Mild digestion with trypsin decreased by approx. 80% the total cell layer-associated HSPG (Table 1), resulting in a major reduction of the peak emerging at K_{av} 0.33, whereas the peaks with K_{av} 0.50 and 0.80 remained practically unchanged, representing intracellular GAG chains and their degradation products respectively [19].

Purified detergent extracts were further submitted to chromatography on octyl-Sepharose CL-4B (Fig. 2B). Comparison of elution profiles indicated that trypsin dramatically decreased the hydrophobic HSPG fraction displaced from gel by 1% Triton X-100 but did not modify the unbound peak which represented intracellular species.

Following trypsin treatment, the lipophilic HSPG

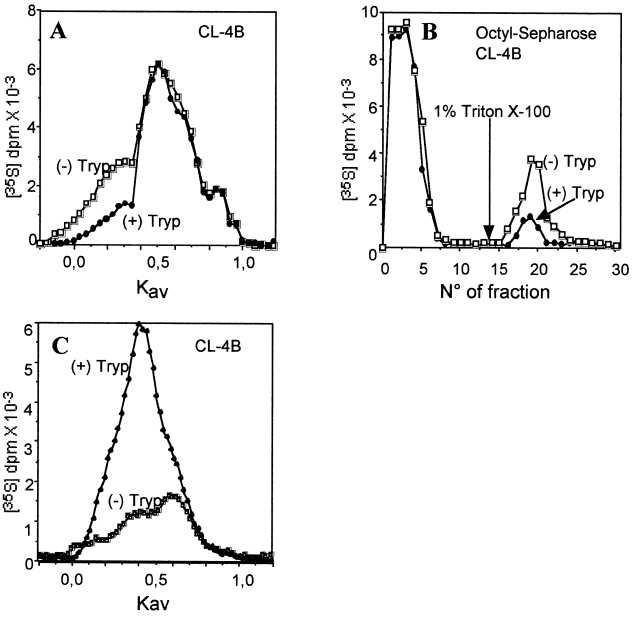

Fig. 2. Trypsin susceptibility of cultured Sertoli cell HSPG. [^{35}S]Sulfate-labeled cell layer Sertoli cells were washed with heparin solution, treated or not (control) with trypsin (100 μg/ml, 3 min, 37°C), then extracted with 0.5% Triton X-100. Extract was purified by DEAE-Trisacryl chromatography and analyzed. Detergent extract from cells submitted (□) or not (●) to mild trypsin digestion was chromatographed (A) on Sepharose CL-4B with 4 M urea buffer as eluent and (B) on octyl-Sepharose CL-4B. Hydrophobic HSPG were eluted by buffer containing 1% Triton X-100. (C) Trypsin-susceptible ^{35}S-labeled integral membrane HSPG released in the incubation medium were DEAE-Trisacryl purified and chromatographed on Sepharose CL-4B (●). Control medium sample (□) was analyzed with the same procedure.

fraction was reduced from 62.5% to 12.5% of the total detergent extract HSPG. The proportion of the total cellular PG released from the cell surface by trypsin accounted for this reduction in cellular lipophilic HSPG. Thus, about 80% of the hydrophobic HSPG are integrated in the plasma membrane, while the remainder (20%) was not accessible to trypsin and presumably located in the intracellular compartment (Table 1).

The trypsin-susceptible ^{35}S-labeled integral membrane PG released in the incubation medium were DEAE-Trisacryl purified, analyzed by gel chromatography on Sepharose CL-4B and compared with those of control culture medium (Fig. 2C). These released HSPG (as revealed following nitrous acid degradation, data not shown) eluted with a $K_{av} = 0.38$ whereas the integral membrane HSPG

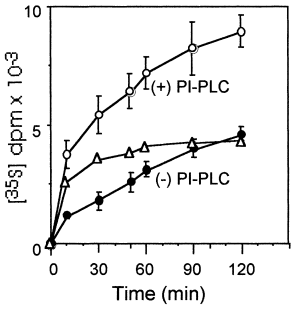

Fig. 3. PI-PLC susceptibility of cultured Sertoli cell HSPG. Time course of GPI-PG release. Sertoli cells were labeled with [^{35}S]sulfate. After removing the labeling medium, cells were incubated in fresh medium without (●, control) or with 0.2 IU/ml PI-PLC (○) for up to 120 min. Aliquots of media were collected at each time point and the amount of [^{35}S]HSPG was measured by quantitative solid phase assay (see Section 2) ($n=3$). The amount of enzymatically released GPI-PG was calculated by subtracting the radioactivity of control cell medium from the radioactivity of PI-PLC-treated cell medium (△).

chromatographed with a $K_{av}=0.33$ (Fig. 1B). After protease treatment, the decrease in HSPG species apparent molecular mass may almost result from removal of membrane insertion and cytoplasmic domains of transmembrane HSPG [32] and from possible proteolytic cleavage affecting some GPI-HSPG, near the C-terminal end of the core protein [33].

3.3. Cell layer PI-PLC-susceptible HSPG

PI-PLC provides a useful experimental tool to demonstrate the presence of GPI molecules and their surface localization [34]. Cells were labeled with [^{35}S]sulfate for 48 h and then incubated in fresh medium with or without PI-PLC (0.2 IU/ml) for up to 120 min, allowing a direct quantification of the GPI-anchored cell surface proteoglycans. [^{35}S]PG released in the medium were measured at 0, 10, 30, 50, 60, 90 and 120 min (Fig. 3). At each time point, [^{35}S]PG radioactivity in the medium was 2–3-fold higher in PI-PLC-treated cells than in control cells. Under our experimental conditions, the totality of the radioactivity associated to GPI-PG was released after 1 h PI-PLC treatment (Fig. 3).

In other experiments, Sertoli cells were double radiolabeled with [^{35}S]sulfate and [^{3}H]inositol for 48 h. Medium was removed, the cell layer was washed with PBS containing heparin (100 mg/ml), then treated or not with PI-PLC (0.2 IU/ml) for 1 h. PG released by the enzymatic treatment were purified by DEAE-Trisacryl chromatography. Radiolabeled molecules secreted in the absence of the enzyme during the same incubation period (control secretion) were similarly chromatographed.

PI-PLC released 16% of the total HSPG present in Sertoli cells (Table 1). After ion-exchange chromatography, material spontaneously secreted was resolved as a unique [^{35}S]sulfate- and [^{3}H]inositol-labeled peak, eluted by 0.4 M NaCl (Fig. 4A). Treatment by PI-PLC resulted in an increase in [^{3}H]inositol and [^{35}S]sulfate radioactivities in this peak (Fig. 4C). The total [^{3}H]inositol radioactivity/total [^{35}S]sulfate radioactivity ratio of the peak from control culture medium and from PI-PLC-treated culture medium was respectively 0.86 and 1.42. This confirms that PI-PLC selectively released GPI-PG.

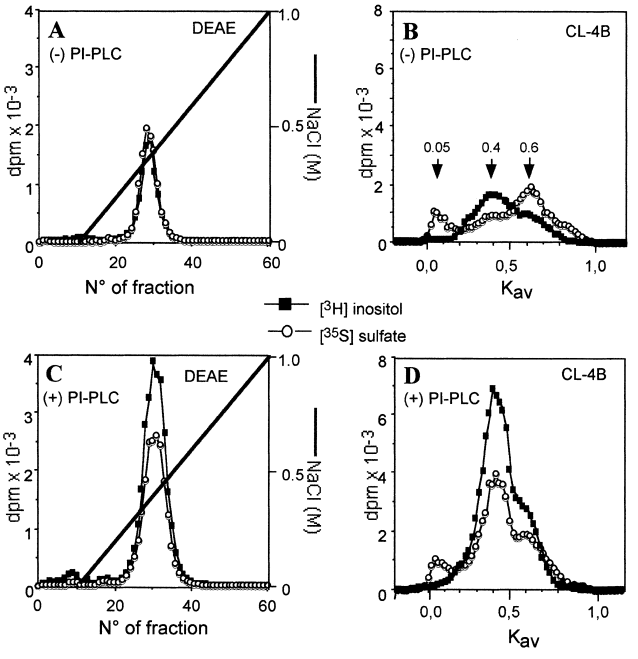

Fig. 4. Analysis of HSPG released in incubation medium after PI-PLC treatment. Sertoli cells were double labeled with [^{35}S]sulfate (○) and [^{3}H]inositol (■) for 48 h; the labeling medium was removed, cells were incubated in fresh medium without (A,B, control) or with 0.2 IU/ml PI-PLC (C,D) for 60 min. Medium HSPG were purified by an anion-exchange DEAE-Trisacryl column (A,C). The [^{35}S]sulfate and [^{3}H]inositol double labeled peak emerging on the DEAE-Trisacryl column (fractions 21–38) was recovered and submitted to gel filtration on Sepharose CL-4B (B,D).

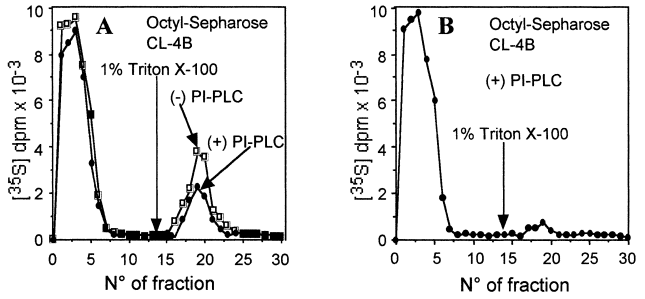

Fig. 5. Hydrophobicity test for HSPG after PI-PLC treatment. [^{35}S]Sulfate-labeled Sertoli cells were washed with heparin solution, treated (●) or not (□, control) with 0.2 IU/ml PI-PLC for 1 h. Incubation medium was recovered and cell layer extracted with 0.5% Triton X-100. Medium and detergent extract were purified by anion exchange on a DEAE-Trisacryl column and chromatographed on octyl-Sepharose CL-4B. Hydrophobic HSPG were eluted with 1% Triton X-100. (A) Triton X-100 extract; (B) PI-PLC incubation medium.

Double labeled PG purified on DEAE-Trisacryl were then analyzed by Sepharose CL-4B gel filtration. For control cells (Fig. 4B), [^{35}S]PG eluted as two main peaks, one at $K_{av} = 0.05$ and the other at $K_{av} = 0.60$ with a discrete shoulder at $K_{av} = 0.40$ coinciding with a [^{3}H]inositol radioactivity peak. PI-PLC specifically released a peak eluted at $K_{av} = 0.40$ of [^{35}S]PG which was also [^{3}H]inositol labeled (Fig. 4D). Peak fractions were pooled, treated with nitrous acid and re-chromatographed on Sepharose CL-4B leading to the emergence of the labeling in the total volume (not shown). These results indicated that the peak eluted at $K_{av} = 0.40$ contained HSPG with a [^{3}H]glycosylphosphatidylinositol anchor. In both control and PI-PLC-treated material, the peak at $K_{av} = 0.60$ was [^{3}H]inositol radiolabeled and susceptible to nitrous acid treatment (data not shown). Due to their apparent molecular mass, the molecular species of this peak may represent degradation products of GPI-PG. The pool containing large PG ($K_{av} = 0.05$) was devoid of [^{3}H]inositol labeling and was not further analyzed.

The PI-PLC-treated cell layer was extracted with Triton X-100 and after DEAE-Trisacryl purification, the extract was submitted to hydrophobic interaction chromatography (Fig. 5A). Compared to control extract, the HSPG fraction bound to the gel was reduced by 30–35%.

Purified HSPG released in the medium by PI-PLC treatment did not bind to octyl-Sepharose (Fig. 5B). This result suggested that the hydrophobic properties of GPI-PG were exclusively due to the presence of the phospholipid anchor.

Coupled results obtained after trypsin and PI-PLC

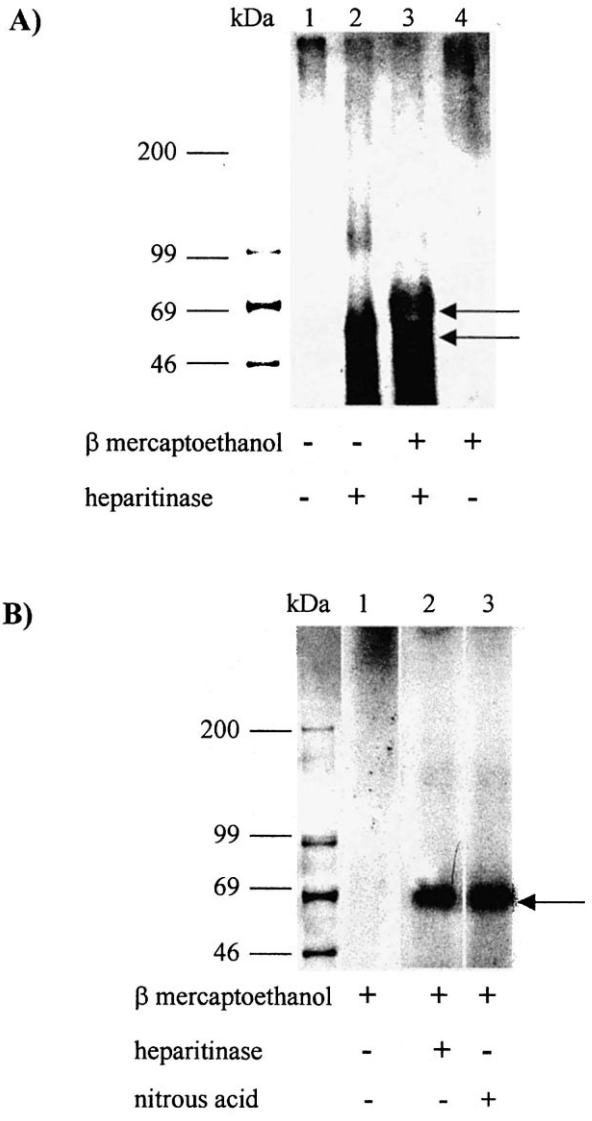

Fig. 6. Core protein electrophoresis of integral membrane HSPG. Sertoli cells were labeled with [^{35}S]sulfate. Integral membrane HSPG were extracted with Triton X-100, purified by anion exchange and hydrophobic interaction chromatographies, then PI-PLC treated. GPI-HSPG and transmembrane HSPG populations were separated after liposome intercalation and Sepharose CL-4B gel filtration. Core proteins were ^{125}I-radiolabeled (see Section 2) and HS chains submitted or not to degradation by heparitinase or nitrous acid. Samples were resolved by gradient SDS-PAGE in the presence or absence of β-mercaptoethanol and visualized by autoradiography. (A) Transmembrane HSPG; (B) GPI-HSPG.

digestion indicated that, in Sertoli cells, transmembrane HSPG and GPI-HSPG represented respectively 68% and 32% of the integral membrane HSPG (Table 1).

3.4. Isolation of hydrophobic HSPG core proteins

GPI-HSPG and transmembrane HSPG were distinguished and separated on the basis of their PI-PLC susceptibility. Their respective core proteins were ^{125}I-radiolabeled, isolated and purified as described in Section 2. ^{125}I-Labeled core proteins were analyzed by SDS-polyacrylamide gel electrophoresis and revealed by autoradiography.

Under reducing or non-reducing conditions and prior to heparitinase treatment, PI-PLC-resistant [^{125}I]HSPG (syndecan species presumed) migrated as a diffuse band in the high molecular mass region (Fig. 6A, lanes 1 and 4). Heparitinase digestion resulted in an important decrease of this band (residual radioactivity beyond 200 kDa probably represented a small amount of undigested PG) and emergence of ^{125}I-labeled bands at 60–68 kDa (Fig. 6A, lanes 2 and 3).

Under reducing conditions and prior to deglycanation, PI-PLC-susceptible [^{125}I]HSPG (glypican species presumed) remained as a smear on the top of the gel (Fig. 6B, lane 1). After heparitinase digestion or nitrous acid treatment, a band with an apparent molecular mass of 64–65 kDa appeared (Fig. 6B, lanes 2 and 3).

4. Discussion

In the present work, HS molecules isolated from cultured immature Sertoli cells were discriminated on the basis of their high anionic charge, hydrodynamic size, lipophilic properties and susceptibility to trypsin and PI-PLC. We have shown that in detergent extract, about 65% of ^{35}S labeling was associated to HS-free chains and their degradation products (Table 1) [19]. Probably, these compounds resulted from the HSPG catabolism pathway and were located in the lysosomal compartment. This localization was suggested by the fact that isolated plasma membrane, partially contaminated by lysosomal components, contained low molecular size [^{35}S]sulfate species emerging in the total volume of Sepharose CL-2B (Fig. 1, peak II). [^{35}S]Sulfate is also incorporated in the sulfated glycoprotein-2 (SGP-2)/clusterin (51.4 kDa) that is specifically produced in the testis, by Sertoli cells [35]. It was shown that another epithelial cell type (non-ciliated cells of efferent ducts) synthesizes an endogenous form of SGP-2 that is targeted to the lysosomal compartment [36]. This process, although not described in Sertoli cells, might be possible. However, nitrous acid practically degraded all peak II, revealing the exclusive presence of [^{35}S]heparan sulfate.

The remaining (35%) was incorporated into HSPG including a non-hydrophobic population (37.5% of total HSPG, Table 1). Non-hydrophobic HSPG may correspond to HSPG in the synthesis process, constitutively devoid of lipophilic domain and destined to secretion [37]. On the other hand, it may represent degraded forms of integral membrane HSPG, especially syndecans, since it was shown that these transmembrane HSPG were both released in medium and endocytosed. After internalization, they were submitted to a slow and stepwise degradation process which generated distinct degradation intermediates [38]. Some of the non-hydrophobic intermediates may derive from partially catabolized syndecans without a membrane insertion domain.

Trypsin releases integral membrane HSPG and separates the core proteins from their hydrophobic anchor which remains plasma membrane associated. Despite trypsin digestion, a minor fraction of detergent extract HSPG were trypsin resistant and retained hydrophobic properties (12.5% of total HSPG and 20% of total hydrophobic HSPG). Trypsin inaccessibility suggested that this HSPG population was not present on the cell surface but located inside the cell. It may consist of neo-synthesized integral membrane HSPG in advance from the Golgi apparatus to the plasma membrane [37].

Hydrophobic trypsin-susceptible HSPG were presumed to be plasma membrane constituents. This localization was further confirmed by analysis of purified Sertoli cell plasma membrane. (i) When detergent-free 4 M guanidine chloride extract from purified plasma membrane was submitted to gel filtration on Sepharose CL-2B, one peak emerged in the void volume of the column and consisted of HSPG aggregates associated to membrane lipids by hydrophobic

interactions; these HSPG-lipid complexes were dissociated after detergent addition and eluted at $K_{av} = 0.33$ on Sepharose CL-4B. (ii) Additional evidence of hydrophobicity of HSPG from plasma membrane extract was provided by chromatography on hydrophobic matrix (octyl-Sepharose CL-4B) and liposome insertion.

Alternatively, the Sertoli cell layer was trypsin treated, then extracted by Triton X-100 and extract was purified and subjected to gel filtration on Sepharose CL-4B. Under these conditions, the HSPG peak eluted at $K_{av} = 0.33$ was decreased by about 80%. In the same way, the hydrophobic HSPG population retained on octyl-Sepharose CL-4B was reduced to about 20% of the control value. These results were in good agreement with those obtained from the experimental quantitative procedure described in Table 1. Moreover, the extracellular domain of the HSPG population released from the cell surface by trypsin was apparently smaller than the parent integral membrane HSPG population ($K_{av} = 0.38$ vs. 0.33 for parent molecules, on Sepharose CL-4B) and was devoid of lipophilic features.

It is known that trypsin cleaves, in vitro, syndecan core proteins at a site adjacent to the membrane insertion domain, which was represented by a dibasic sequence in the syndecan-1, -2 and -3 and a basic residue in the syndecan-4 [39]. The syndecan ectodomain was released in the trypsin incubation medium whereas transmembrane and cytoplasmic domains were always embedded in the plasma membrane. Furthermore, the primary structure of six known glypicans included basic sequences susceptible to trypsin, some of them located between the fixation sites of HS chains and the plasma membrane [10,11]. A more specific study of glypican was achieved by the use of PI-PLC which releases the whole HSPG with the terminal phosphate group of inositol still attached, whereas diacylglycerol remains associated to the external leaflet of the membrane. Addition of the lipase to the Sertoli cell layer induced, in the incubation medium, a rapid and time-dependent accumulation of HSPG having lost their hydrophobic properties. Furthermore, PI-PLC susceptible HSPG incorporated inositol which is a characteristic residue of the GPI anchor. These results indicated the presence of glypican on the Sertoli cell surface which represented about one third of integral membrane HSPG (Table 1).

The hydrophobic features of detergent-solubilized cellular HSPG, their isolation from purified plasma membranes, their release by phospholipase C and, more extensively, by mild trypsin treatment, strongly suggested an integral membrane disposition and the possible presence of glypican and syndecan species.

Furthermore, PG from the medium, cell periphery and extracellular matrix revealed no hydrophobic properties since they were unable to insert into liposomes (data not shown).

Electrophoresis of hydrophobic HSPG core proteins, susceptible or resistant to PI-PLC, revealed the existence of two integral membrane HSPG families in immature Sertoli cells. A band of 64–65 kDa corresponding to GPI-HSPG was observed that might represent glypican-1: (i) by the RT-PCR technique, we have shown that immature Sertoli cells expressed at least glypican-1 mRNA [40]; (ii) its expression, although observed in cells of endothelial and mesenchymal origin, is prevalent in polarized epithelial cells [41]; and (iii) glypican-2 to -6 were absent or weakly expressed in mammalian testis [11,42,43]. However, the confirmation of glypican identity remains to be established by the use of specific monoclonal antibody.

Glycosidase-digested syndecans members, submitted to electrophoresis in SDS-polyacrylamide gel, migrated as bands corresponding to 2 and 4 times the predicted molecular mass of monomeric protein. Indeed, all syndecans seem to be able to form SDS-resistant oligomers, especially dimers [5]. In accordance with these observations, our results have shown that core proteins of PI-PLC-resistant hydrophobic HSPG localized in electrophoretogram regions where size markers are detected with a higher molecular mass than any known syndecan [39]. Moreover, the reduction of samples by the addition of β-mercaptoethanol did not modify the electrophoretic profile. Recent studies have confirmed that syndecan core proteins can self-associate and that multimerization requires different core protein domains depending on the syndecan isoforms, as described for syndecan-2 [44,45] and syndecan-3 [46]. In vivo, self-association of syndecan core proteins was speculated to correlate with the recruitment of cytoskele-

ton proteins and initiation of signaling pathway activation [5,14]. Since syndecan-1 and syndecan-4 mRNAs were present in immature Sertoli cells [40], these bands may correspond at least to syndecan-1 and-4 core proteins. However, the existence of other glypican or syndecan isoforms, not examined, cannot be ruled out. These results should be considered by further monoclonal antibody approach.

The implication of integral membrane HSPG in biological functions of Sertoli cells is still elusive. Sertoli cells are the target of numerous endocrine and paracrine factors originating from pituitary gland (FSH) and testis cells such as peritubular cells, Leydig cells and germ cells [47]. As coreceptors, syndecans and glypicans sequester soluble ligands, modulate their activity and control the assembly of the resulting signaling complex. Previous work has shown that undersulfation of Sertoli cell surface HSPG induced enhancement of FSH-stimulated estradiol synthesis [25] but did not modify the interaction between the FSH receptor and its ligand (data not shown). This latter observation contrasts strongly with the effects resulting from the decrease in HS sulfate charge on the complex formation of growth factors, chemokines and cytokines with their respective receptors [5,48,49]. However, indirect evidence suggested that increased estradiol production is due, for one part, to the persistence of a high cAMP level following the decrease in cAMP phosphodiesterase activity. It was postulated that alteration of catalytic capacities might be the consequence of the enzyme translocation from cytosol to plasma membrane [25]. If directly confirmed, transmembrane HSPG involvement in subcellular localization of cAMP phosphodiesterase might be considered a reminiscence of protein kinase C recruitment and activity regulation by the cytoplasmic variable domain of syndecan-4 [44].

In testis, Sertoli cells play highly diversified roles by participating in the maintenance of seminiferous tubule integrity through interactions with peritubular cells and providing structural support for spermatogenesis. Many of these events, mediated by soluble and insoluble ligands, probably require HSPG collaboration. Whether each of the integral membrane HSPG forms is devolved to specific function(s) remains to be cleared up.

Acknowledgements

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References

[1] M. Salmivirta, K. Lidholt, U. Lindahl, Heparan sulfate: a piece of information, FASEB J. 10 (1996) 1270–1279.
[2] U. Lindahl, M. Kusche-Gullberg, L. Kjellen, Regulated diversity of heparan sulfate, J. Biol. Chem. 273 (1998) 24979–24982.
[3] R.V. Iozzo, Matrix proteoglycans: from molecular design to cellular function, Annu. Rev. Biochem. 67 (1998) 609–652.
[4] W. Halfter, S. Dong, B. Schurer, G.J. Cole, Collagen XVIII is a basement membrane heparan sulfate proteoglycan, J. Biol. Chem. 273 (1998) 25404–25412.
[5] M. Bernfield, M. Gotte, W.P. Park, O. Reizes, M.L. Fitzgerald, J. Lincecum, M. Zako, Functions of cell surface heparan sulfate proteoglycans, Annu. Rev. Biochem. 68 (1999) 729–777.
[6] F. Lopez-Casillas, S. Cheifetz, J. Doody, J.L. Andres, W.S. Lane, J. Massague, Structure and expression of the membrane proteoglycan betaglycan, a component of the TGF-beta receptor system, Cell 67 (1991) 785–795.
[7] X.F. Wang, H.Y. Lin, E. Ng-Eaton, J. Downward, H.F. Lodish, R.A. Weinberg, Expression cloning and characterization of the TGF-beta type III receptor, Cell 67 (1991) 797–805.
[8] D.G. Jackson, J.I. Bell, R. Dickinson, J. Timans, J. Shields, N. Whittle, Proteoglycan forms of the lymphocyte homing receptor CD44 are alternatively spliced variants containing the v3 exon, J. Cell Biol. 128 (1995) 673–685.
[9] K.L. Bennett, D.G. Jackson, J.C. Simon, E. Tanczos, R. Peach, B. Modrell, I. Stamenkovic, G. Plowman, A. Aruffo, CD44 isoforms containing exon V3 are responsible for the presentation of heparin-binding growth factor, J. Cell Biol. 128 (1995) 687–698.
[10] M. Veugelers, G. David, The glypicans: a family of GPI-anchored heparan sulfate proteoglycans with a potential role in the control of cell division, Trends Glycosci. Glycotechnol. 10 (1998) 145–152.
[11] M. Veugelers, B. De Cat, H. Ceulemans, A.M. Bruystens, C. Coomans, J. Durr, J. Vermeesch, P. Marynen, G. David, Glypican-6, a new member of the glypican family of cell surface heparan sulfate proteoglycans, J. Biol. Chem. 274 (1999) 26968–26977.
[12] E. Ruoslahti, Structure and biology of proteoglycans, Annu. Rev. Cell Biol. 4 (1988) 229–255.
[13] M.C. Farach-Carson, D.D. Carson, Extraction and isolation of glycoproteins and proteoglycans, BioTechniques 7 (1989) 482–493.

[14] P. Zimmermann, G. David, The syndecans, tuners of transmembrane signaling, FASEB J. 13 (1999) S91–S100.
[15] M.D. Griswold, The central role of Sertoli cells in spermatogenesis, Cell Dev. Biol. 9 (1998) 411–416.
[16] M.K. Skinner, P.S. Tung, I.B. Fritz, Cooperativity between Sertoli cells and testicular peritubular cells in the production and deposition of extracellular matrix components, J. Cell Biol. 100 (1985) 1941–1947.
[17] M.K. Skinner, I.B. Fritz, Structural characterization of proteoglycans produced by testicular peritubular cells and Sertoli cells, J. Biol. Chem. 260 (1985) 11874–11883.
[18] L. Bichoualne, B. Thiébot, M. Langris, P. Barbey, H. Oulhaj, J. Bocquet, Membrane associated proteoglycans in rat testicular peritubular cells, Mol. Cell. Biochem. 140 (1994) 37–48.
[19] A. Mounis, P. Barbey, M. Langris, J. Bocquet, Detergent-solubilized proteoglycans in rat testicular Sertoli cells, Biochim. Biophys. Acta 1074 (1991) 424–432.
[20] C. Mauduit, M. Benhamed, Growth factors in testis development and function, in: S. Hamamah, R. Mieusset (Eds.), Research in Male Gametes: Production and Quality, Editions INSERM, Paris, 1996, pp. 3–45.
[21] L. Malcom, J.T. Gallaher, Bio-specific sequences and domains in heparan sulphate and the regulation of cell growth and adhesion, Matrix Biol. 17 (1998) 485–493.
[22] I. Masayuki, O. Katsuaki, Structure and function of heparin and heparan sulfate; heparinoid library and modification of FGF-activities, Trends Glycosci. Glycotechnol. 10 (1998) 223–233.
[23] E. Piek, C.H. Heldin, P.T. Dijke, Specificity, diversity, and regulation in TGF-β superfamily signaling, FASEB J. 13 (1999) 2105–2124.
[24] N.T. Phamantu, P.J. Bonnamy, M. Bouakka, J. Bocquet, Inhibition of proteoglycan synthesis induces an increase in follicle stimulating hormone (FSH)-stimulated estradiol production by immature rat Sertoli cells, Mol. Cell. Endocrinol. 109 (1995) 37–45.
[25] N.T. Phamantu, G. Fagnen, F. Godard, J. Bocquet, P.J. Bonnamy, Sodium chlorate induces undersulfation of cellular proteoglycans and increases in FSH-stimulated estradiol production in immature rat Sertoli cells, J. Androl. 20 (1999) 241–250.
[26] P.S. Tung, M.K. Skinner, I.B. Fritz, Fibronectin synthesis is a marker for peritubular contaminants in Sertoli cell-enriched cultures, Biol. Reprod. 30 (1984) 199–211.
[27] M. Galdieri, E. Ziparo, F. Palombi, M.A. Russo, M. Stephanini, Pure Sertoli cultures: a new model for the study of somatic-germ cell interactions, J. Androl. 2 (1981) 249–254.
[28] V. Lories, G. David, J.J. Cassiman, H. Van Den Berghe, Heparan sulfate proteoglycans of the human lung fibroblasts. Occurrence of distinct membrane, matrix and secreted forms, Eur. J. Biochem. 158 (1986) 351–360.
[29] M.M. Bradford, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Anal. Biochem. 72 (1976) 248–254.
[30] A. Rapraeger, C. Yeaman, A quantitative solid-phase assay for identifying radiolabeled glycosaminoglycans in crude cell extracts, Anal. Biochem. 179 (1989) 361–365.
[31] G.W. Conrad, C. Hamilton, E. Haynes, Differences in glycosaminoglycans synthesized by fibroblast-like cells from chick cornea, heart, skin, J. Biol. Chem. 252 (1977) 6861–6870.
[32] A. Rapraeger, M. Bernfield, Cell surface proteoglycan of mammary epithelial cells. Protease releases a heparan sulfate-rich ectodomain from a putative membrane-anchored domain, J. Biol. Chem. 260 (1985) 4103–4109.
[33] G. David, V. Lories, B. Decock, P. Marynen, J.J. Cassiman, H. Van den Berghe, Molecular cloning of a phosphatidylinositol-anchored membrane heparan sulfate proteoglycan from human lung fibroblasts, J. Cell Biol. 111 (1990) 3165–3176.
[34] G. Müller, A. Korndorfer, K. Saar, B. Karbe-Thonges, H. Fasold, S. Mullner, 4′-Amino-benzamido-taurocholic acid selectively solubilizes glycosyl-phosphatidylinositol-anchored membrane proteins and improves lipolytic cleavage of their membrane anchors by specific phospholipases, Arch. Biochem. Biophys. 309 (1994) 329–340.
[35] M.W. Collard, M.D. Griswold, Biosynthesis and molecular cloning of sulfated glycoprotein 2 secreted by rat Sertoli cells, Biochemistry 26 (1987) 3297–3303.
[36] S.A. Igdoura, L. Hermo, C.R. Morales, Sulfated glycoprotein-2 synthesized by nonciliated cells of efferent ducts is targeted to the lysosomal compartment, Microsc. Res. Tech. 29 (1994) 468–480.
[37] J.E. Silbert, G. Sugumaran, Intracellular membranes in the synthesis, transport, and metabolism of proteoglycans, Biochim. Biophys. Acta 1241 (1995) 371–384.
[38] M. Yanagishita, Cellular catabolism of heparan sulfate proteoglycans, Trends Glycosci. Glycotechnol. 10 (1998) 57–63.
[39] M. Okayama, S. Munesue, M. Komaki, Y. Kusano, K. Oguri, Functional specificity of syndecans, Trends Glycosci. Glycotechnol. 10 (1998) 211–221.
[40] S. Brucato, A. Harduin-Lepers, F. Godard, J. Bocquet, C. Villers, Expression of glypican-1, syndecan-1 and syndecan-4 mRNAs protein kinase C-regulated in rat immature Sertoli cells by semi-quantitative RT-PCR analysis, Biochim. Biophys. Acta 1474 (2000) 31–40.
[41] G. Mertens, B. Van der Schueren, H. van den Berghe, G. David, Heparan sulfate expression in polarized epithelial cells: the apical sorting of glypican (GPI-anchored proteoglycan) is inversely related to its heparan sulfate content, J. Cell Biol. 132 (1996) 487–497.
[42] S. Saunders, S. Paine-Saunders, A.D. Lander, Expression of the cell surface proteoglycan glypican-5 is developmentally regulated in kidney, limb, and brain, Dev. Biol. 190 (1997) 78–93.
[43] M. Pellegrini, G. Pilia, S. Pantano, F. Lucchini, M. Uda, M. Fumi, A. Cao, D. Schlessinger, A. Forabosco, Gpc3 expression correlates with the phenotype of the Simpson-Golabi-Behmel syndrome, Dev. Dyn. 213 (1998) 431–439.
[44] E.S. Oh, A. Woods, J.R. Couchman, Multimerization of the

cytoplasmic domain of syndecan-4 is required for its ability to activate protein kinase C, J. Biol. Chem. 272 (1997) 11805–11811.

[45] E.S. Oh, J.R. Couchman, A. Woods, Serine phosphorylation of syndecan-2 proteoglycan cytoplasmic domain, Arch. Biochem. Biophys. 344 (1997) 67–74.

[46] V.K. Asundi, D.J. Carey, Self-association of N-syndecan (syndecan-3) core protein is mediated by a novel structural motif in the transmembrane domain and ectodomain flanking region, J. Biol. Chem. 270 (1995) 26404–26410.

[47] M.K. Skinner, Cell-cell interactions in the testis, Endocr. Rev. 12 (1991) 45–77.

[48] G.S.V. Kuschert, F. Coulin, C.A. Power, A.E.I. Proudfoot, R.E. Hubbard, A.J. Hoogewerf, T.N.C. Wells, Glycosaminoglycans interact selectively with chemokines and modulate receptor binding and cellular responses, Biochemistry 38 (1999) 12959–12968.

[49] Y. Tanaka, K. Kimata, D.H. Adams, S. Eto, Modulation of cytokine function by heparan sulfate proteoglycans: sophisticated models for the regulation of cellular responses to cytokines, Proc. Assoc. Am. Phys. 110 (1998) 118–125.