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The heparin-binding lectin from ovine placenta: Purification and identification as histone H4

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The heparin-binding lectin complex from ovine placental cotyledons was purified by affinity chromatography on heparin-agarose column. It showed three protein bands, which had molecular weights of 13 000, 15 000 and 17 000 by sodium dodecylsulfate-polyacrylamide gel electrophoresis, and the presence of DNA by agarose gel electrophoresis. The protein components of the complex were separated by reverse-phase HPLC. The minimum inhibitory concentrations of glycosaminoglycans were significantly different for the lectin complex and the separated proteins, suggesting affinity changes upon DNA binding. The haemagglutinating activity specificity allowed the characterization of the fraction with a molecular weight of 13 000 as the heparin-binding lectin. This protein was identified as histone H4 by internal sequencing, thus showing that this is the histone responsible for the heparin-binding property of the complex. The accompanying proteins were tentatively identified as histones H2A and H2B.

***Keywords*: lectin, heparin, placental, ovine, heparin-binding protein, histone**

Introduction

Heparin and heparan sulfate are highly negatively charged glycosaminoglycans, which are involved in a great number of cellular functions [1], like growth modulation [2], mammalian fertilization [3], angiogenesis [4], inhibition of HIV-1 infectivity [5], etc. They exert these functions through their interaction with various cellular components, such as growth factors [6], enzymes [7], transcription factors [8], extracellular matrix proteins [1], blood coagulation factors [9], etc. The possibility of its implication in biosignalling through its interaction with cellular receptors has been strongly suggested [10]. Embryo attachment was inhibited by their treatment with heparin/heparan sulfate lyases or inhibitors of proteoglycan synthesis [11, 12], and this finding prompted the isolation of an heparin/heparan sulfate binding protein from human uterine epithelial cells [13].

A heparin-inhibitable lectin activity was found in chicken [14], rat tissues [15] and in human placenta [16]. Kohnke-Godt and Gabius purified and partially characterized a heparin-binding lectin from human placenta [10, 17]. They found an aminoterminal sequence homology with histone H2B, and an antigenic relation to histones, although they report that the lectin is a distinct molecular entity from histones H2A, H2B, the fibroblast growth factor and angiogenin.

In this article we describe the purification of the heparin-binding lectin from ovine placenta, and its characterization as a complex of three histones and DNA. Further dissociation of this complex and isolation of the individual histones allowed the assignation of the heparin-binding capacity to histone H4.

Materials and methods

Heparin-agarose, phenylmethylsulfonyl fluoride (PMSF), bovine albumin, dithiothreitol, β-mercaptoethanol, molecular weight markers, D-lactose, D-glucose, D-fucose, D-galactose, D-mannose, melibiose, cellobiose, *N*-acetyl-D-glucosamine, *N*-acetylglucosamine-3-sulfate, D-fructose-1,6-biphosphate, D-galactose-6-phosphate, D-galacturonic acid, polygalacturonic acid, heparin, hyaluronic acid, *N*-acetylneuraminic acid, carrageenan λ and κ, chondroitin sulfate A and B, fucoidan, dextran sulfate, histone type II-S from calf thymus and trypsin were purchased from Sigma Chemical Co. (St Louis, MO, USA) and trifluoroacetic acid from Baker Chemical Co. (Phillipsburg, NJ, USA). Acetonitrile was HPLC grade and all other chemicals were AR grade.

Ovine placental tissue was surgically removed from ewes at a slaughterhouse, immediately frozen and stored at $-70\,^{\circ}C$ prior to processing.

Haemagglutination and haemagglutination inhibition assays

Rabbit blood was obtained by venipuncture. Erythrocytes were treated with trypsin and glutaraldehyde [18]. Serial

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dilutions (two-fold) of the protein solution in 10 mM Na phosphate buffer, 0.15 M NaCl, pH 7.0 (25 µl) were mixed with equal volumes of 1% bovine albumin and of 2.5% suspension of treated erythrocytes in the same buffer. After incubation for 1 h at room temperature, the agglutination was recorded visually. The reciprocal of the highest dilution of protein solution showing visible agglutination was recorded as the titre. Haemmagglutination inhibition assays were carried out as previously described [19].

Acetonitrile was evaporated from the fractions obtained by HPLC and then each fraction was diluted two-fold with 10 mM Na phosphate buffer, 0.15 M NaCl, pH 7.0. After incubation for 30 min at 4 °C the haemagglutination and haemagglutination inhibition assays were carried out as described above.

Purification of heparin-binding lectin from ovine placental cotyledons

All steps were performed at 4 °C unless otherwise stated. Routinely, 20 g of frozen ovine placental cotyledons were homogenized for 1 min in an Omnimixer in 40 ml of 20 mM Na phosphate buffer, 0.5 M NaCl, 2 mM EDTA, 2 mM DTT, pH 7.2 (buffer A), also containing 1 mM PMSF and 0.1 M lactose [17]. The homogenate was centrifuged at $20\,000 \times \mathbf{g}$ for 30 min. The resulting supernatant was further ultracentrifuged at $105\,000 \times \mathbf{g}$ for 90 min. This latest supernatant was applied slowly to a heparin-agarose column (1.5 × 15 cm) previously equilibrated with buffer A. After having been thoroughly washed with the same buffer, the column was washed with 10 mM Tris-HCl buffer, 2 mM DTT, pH 8.6 (buffer B) containing 0.1 M NaCl in order to eliminate the proteins retained in the column with low affinity. The elution was performed with a NaCl gradient in buffer B, raising the salt concentration from 0.2 to 2 M. The fractions were monitored for protein content at 280 nm and for haemagglutinating activity.

The active fractions were pooled, and further separation was achieved by reverse-phase HPLC on a Vydac C_4 column (4.6 × 250 mm), which had been equilibrated with 30% [v/v] acetonitrile, 0.12% [v/v] TFA in water (solvent A). The column was eluted at a flow rate of 0.7 ml min^{-1} with a linear gradient of solvent B (65% [v/v] acetonitrile, 0.1% [v/v] TFA in water).

Protein determination

Protein content was determined by the method of Bradford [20].

Polyacrylamide gel electrophoresis (PAGE)

Dialysed and freeze-dried samples were subjected to PAGE in the presence of sodium dodecyl sulfate (SDS) at room temperature in slab gels as described by Schagger *et al.* [21]. The gels were stained with Coomassie Brilliant Blue R-250.

Agarose gel electrophoresis

It was performed as described by Sambrook *et al.* [22], in 1% agarose gels and 45 mM Tris-borate buffer, 1 mM EDTA. The gels were stained with ethidium bromide.

Gel filtration analysis

Samples were submitted to FPLC at room temperature on a Superose 12 HR 10/30 column (Pharmacia LKB) calibrated with standard proteins. The column was eluted with 10 mM Na phosphate buffer, 0.15 M NaCl, pH 7.0 at a flow rate of 0.5 ml min^{-1}. The eluent was monitored for protein content at 280 nm and for haemagglutinating activity.

Tryptic digestion

The proteins were reduced, carbamidomethylated and digested with trypsin at 1:20 enzyme-to-substrate ratio in 2 M urea, 0.1 M ammonium bicarbonate, at 37 °C for 20 h [23].

Purification of tryptic peptides

These fragments were separated by HPLC on a Brownlee C_{18} column (2.1 × 220 mm) equilibrated with 95% solvent A, 5% solvent B (solvent A, 0.1% [v/v] TFA in water; solvent B, 80% [v/v] acetonitrile, 0.08% [v/v] TFA in water). Elution was performed at a flow rate of 0.8 ml min^{-1} with a 10–60% solvent B gradient in 70 min.

Peptide sequencing

Selected tryptic peptides were applied to a polybrene-coated glass filter and sequenced in an Applied Biosystems Model 477 Automatic Sequencer (Applied Biosystems, Foster City, CA, USA), run according to the manufacturer's instructions.

Results

Purification of the heparin-binding lectin

Affinity chromatography on heparin-agarose column (Figure 1) of a homogenate of 20 g of ovine placental cotyledons yielded a fraction with haemagglutinating activity. This amounted to approximately 5 mg of protein. The fraction was examined by SDS-PAGE and the results are shown on Figure 2. It showed three bands, which had molecular weights of 13 000, 15 000 and 17 000 respectively, and were not affected by the presence of β-mercaptoethanol. Their yield and relative proportions varied from batch to batch, as already noted for other heparin-binding lectins [14, 17]. Spectrophotometric measurements suggested the presence of DNA in this fraction, which was confirmed by agarose gel electrophoresis (data not shown).

When this fraction was submitted to gel filtration on a Superose 12 HR column, it eluted as a single peak corresponding to a molecular weight above 300 000, which retained the haemagglutinating activity.

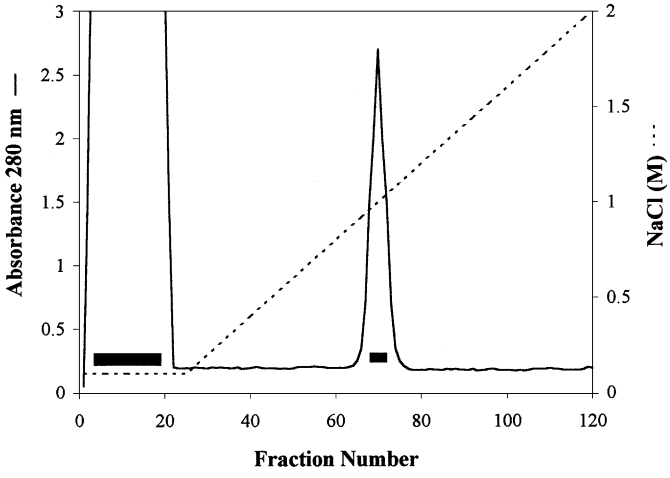

Figure 1. Isolation of the heparin-binding lectin by affinity chromatography on heparin-agarose. The 105 000 g supernatant (see Material and Methods section) was applied to a heparin-agarose column (1.5 × 15 cm) previously equilibrated with 20 M Na phosphate buffer, 0.5 mM NaCl, 2 mM EDTA, 2 mM DTT, pH 7.2. The elution was performed with a 0.2–2 M NaCl gradient in 10 mM Tris-HCl buffer, 2 mM DTT, pH 8.6 (dotted line) and fractions of 2.5 ml were collected. The fractions were monitored for protein content at 280 nm (solid line). The horizontal bars indicate the fractions presenting haemagglutinating activity.

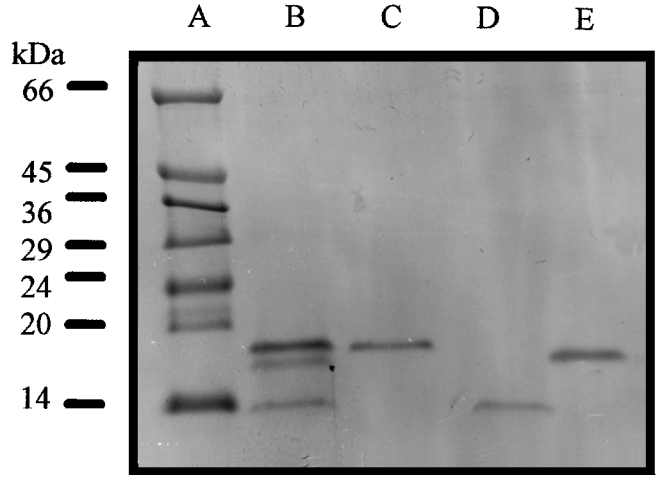

Figure 2. SDS-PAGE of fractions obtained by affinity chromatography and by HPLC. Electrophoresis was carried out on 10% acrylamide gel, as described in the Materials and methods section. Lane A contains 2 μg of each of the following molecular mass markers: bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-P-dehydrogenase (36 kDa), bovine carbonic anhydrase (29 kDa), bovine pancreas trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa) and bovine milk *α*-lactalbumin (14.2 kDa). Lane B, 4 μg of the fraction presenting haemagglutinating activity eluted from the heparin-agarose column. Lanes C, D and E correspond to peaks A, B and C respectively of the HPLC elution profile (Figure 3). The proteins were visualized by Coomasie blue staining. The numbers on the left indicate the position and mass of the protein markers expressed in kDa.

Further separation was achieved by HPLC reverse phase chromatography on a C4 column (Figure 3), which yielded three peaks. When these peaks were analysed by SDS-PAGE, each corresponded to one of the three protein bands. They all presented haemagglutinating activity and did not reveal any band in the agarose gel staining with ethidium bromide thus indicating they did not contain any nucleic acid (data not shown).

Binding specificity

The minimum concentration of various substances for complete inhibition of two haemagglutinating doses of lectin was determined for the complex and for each of the separated peaks (Table 1). Neither uncharged sugars, such as glucose, galactose, fucose, mannose, lactose, melibiose, nor charged sugars such as galacturonic acid, *N*-acetyl-D-glucosamine, *N*-acetylglucosamine-3-sulfate, fructose-1,6-biphosphate, galactose-6-phosphate and *N*-acetylneuraminic acid, were inhibitory at 100 mM. Carrageenan *λ* and fucoidan were the most potent inhibitors of the haemagglutinating activity of the lectin complex, followed by heparin, carrageenan-*κ*, dextran sulfate and polygalacturonic acid, while hyaluronic acid, chondroitin sulfate A and B were weaker inhibitors. Fraction B was the only fraction significantly inhibited by heparin. Besides, it was the only fraction affected by the same inhibitors as the complex, although the minimum inhibitory concentration of each inhibitor was not the same. In some cases there were changes even in the order of potency of the inhibitors.

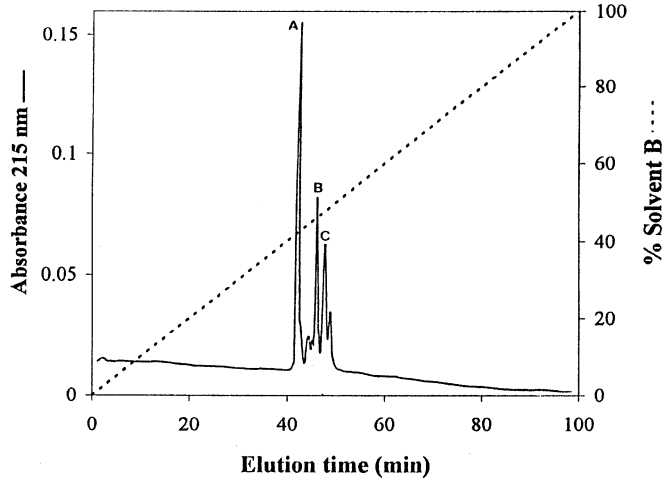

Figure 3. Final purification of the heparin-binding lectin from ovine placenta by reverse-phase HPLC. Fractions presenting haemagglutinating activity eluted from the affinity column (Figure 1) were pooled and loaded on a Vydac C_4 column (4.6 × 250 mm), which had been equilibrated with 30% [v/v] acetonitrile, 0.12% [v/v] TFA in water (solvent A). Elution was performed with a linear gradient of solvent B (65% [v/v] acetonitrile, 0.1% [v/v] TFA in water) that is indicated by the dotted line. The flow rate was 0.7 ml min^{-1}. The eluent was monitored at 215 nm (solid line).

Table 1. Haemagglutination inhibition assay of the lectin complex and fractions A, B and C (Figure 3)

Inhibitor	*Lectin Complex*	*Fraction*		
		A	*B*	*C*
	Minimum inhibitory concentration[a] ($\mu g\ ml^{-1}$)			
Heparin	0.32	NI	4	2000
Fucoidan	0.02	2	0.5	12.5
Carrageenan λ	0.02	0.5	0.13	0.5
Carrageenan κ	1.25	0.2	0.5	0.1
Dextran sulfate	5.25	3.13	0.2	6.25
Hyaluronic acid	31.25	250	12.5	12.5
Chondroitin sulfate A	125	NI	100	2000
Chondroitin sulfate B	31.25	NI	50	NI
Polygalacturonic acid	5.25	400	10	100

[a] Concentration for complete inhibition of two haemagglutinating doses of lectin. NI, no inhibition.

The binding of heparin was not dependent on the presence of Ca^{2+} ions, since the haemagglutination was not affected by the presence of EDTA.

Identification of the proteins

When the three bands revealed by SDS-PAGE were blotted onto a polyvinylidene difluoride membrane and applied to the automatic sequencer, no PTH-amino acid peaks were detected in significant amounts, thus indicating that the N-terminus was blocked.

In order to identify the separated proteins, each of them was subjected to tryptic digestion for internal amino acid sequencing. The peptides obtained were isolated by reverse phase HPLC on a C18 column. The results are shown in Figure 4. Selected peptides were applied to the automatic sequencer and their sequences are shown in Figures 5 and 6 together with the sequences that belong to their homologous proteins. These results show that the peptides from peaks A, B and C are homologous to those from histones H2B, H4 and H2A respectively.

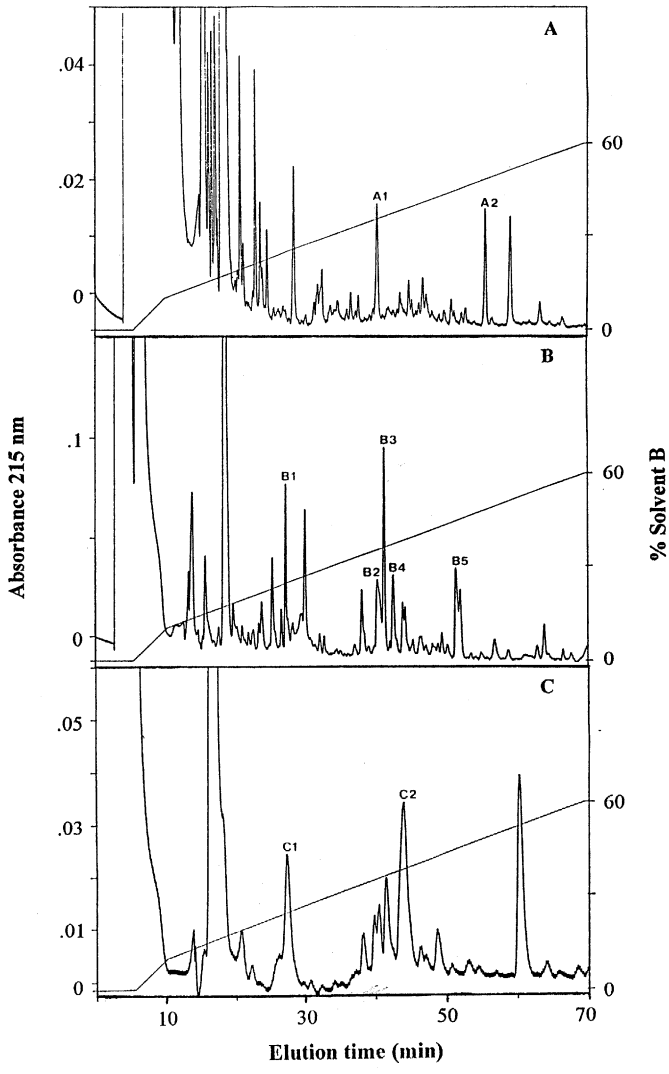

Figure 4. HPLC elution profile of tryptic fragments. Fractions A, B and C obtained by HPLC (Figure 3) were digested with trypsin. Each of the peptide mixtures was separated on a Brownlee C_{18} column (2.1 × 220 mm) equilibrated with 95% solvent A, 5% solvent B (solvent A, 0.1% [v/v] TFA in water; solvent B, 80% [v/v] acetonitrile, 0.08% [v/v] TFA in water). A linear gradient from 10 to 60% solvent B was used, the flow rate was 0.8 ml min^{-1}, and the eluent was monitored at 215 nm. Panels A, B and C correspond to digests from peaks A, B and C (Figure 3) respectively.

Discussion

The fraction with haemagglutinating activity that eluted from the heparin-agarose column, was characterized as a high molecular weight complex formed by three proteins and DNA. It behaved as a single peak on gel filtration, but it dissociated in the presence of SDS and the TFA used in the HPLC solvent, thus showing that these proteins are not covalently bound.

The ligand-binding specificity, as explored by the haemagglutination inhibition assays, appears to be similar to that of the heparin-binding lectin isolated from human placenta [17]. None of the monosaccharides tested, including several phosphorylated sugars, sialic acid and galacturonic acid, showed an inhibitor potency. Consequently, the binding does not appear to be due to charge interactions, although the presence of clusters of negative charges, such as in polygalacturonic acid, produced a weak inhibition.

The dissociation of the complex in the HPLC conditions allowed the isolation of the three protein components. Once separated, all three fractions retained haemagglutinating

Peptide A1 ^{99}L L L P G E L A K^{107}
Histone H2B **L L L P G E L A K**

Peptide A2 ^{57}A M G I M N S F V N D I F E R^{71}
Histone H2B **A M G I M N S F V N D I F E R**

Peptide C1 ^{77}I I P R^{80}
Histone H2A **I I P R**

Peptide C2 ^{42}V G A G A P V Y L R^{51}
Histone H2A **V G A G A P V Y L** A

Figure 5. Sequence of the tryptic peptides from fractions A and C (Figure 4). The sequences of the different peptides are aligned with the sequence of human histones H2B and H2A and the numbering corresponds to the two latter. The nomenclature of the peptides is consistent with that of Figure 4. Bold type is used for residues identical to those in the sequenced peptides.

Peptide B1 ^{68}D A V T Y^{72}
Histone H4 **D A V T Y**

Peptide B2 ^{24}D N I Q G I T K P A I R^{35}
Histone H4 **D N I Q G I T K P A I R**

Peptide B3 ^{96}T L Y G F G^{101}
Histone H4 **T L Y G F G**

Peptide B4 ^{46}I S G L I Y E E T R^{55}
Histone H4 **I S G L I Y E E T R**

Peptide B5 ^{60}V F L E N V I R^{67}
Histone H4 **V F L E N V I R**

Figure 6. Internal sequences of the heparin-binding lectin from ovine placenta. The sequences of the peptides are aligned with the sequence of human histone H4 and the numbering corresponds to the latter, showing complete sequence identity. The nomenclature of the peptides is consistent with that of Figure 4.

activity. The activity of the peak with an apparent molecular weight of 13 000 (peak B, Figure 3) proved to be the most sensitive to heparin, fucoidan, carrageenan λ, κ and dextran sulfate inhibition. Since its amino-terminal end appeared to be blocked, it was necessary to obtain internal sequences in order to identify this protein. The sequence of five peptides, corresponding to 40% of the molecule, proved to be identical to that of histone H4. Since these peptides belong to different regions of the molecule, it can be safely assumed that this is indeed histone H4. On the other hand, the accompanying proteins were characterized by the same procedure. The sequence homology of two peptides from peak A to histone H2B and of two from peak C with histone H2A permits their tentative identification.

In this respect, it is interesting to note that Kohnke-Godt and Gabius [10] described how the amino-terminal sequence of one of the components of the human placental heparin-binding lectin was similar to that of histone H2B, and that there was immunological cross-reactivity between the lectin and histones. They also reported that commercially available histones H2A and H2B had less affinity for heparin than the lectin they isolated. Also, Bilozur and Biswas [24] isolated heparan sulfate-binding proteins from human lung carcinoma cells and characterized two of them as closely related or identical to histones H2A and H2B.

Histone H4 is not commercially available, and it was isolated from the histone mixture obtained from calf thymus by the procedure of Nomoto and Iwai [25]. It showed no heparin-binding capacity nor haemagglutinating activity. In order to examine if this inactivation was caused by the isolation procedure, our lectin (peak B, Figure 3) was subjected to the same conditions and it suffered the same loss of binding capacity. Hence, this procedure precludes any comparison of these properties. However, both proteins eluted in the same position by reverse phase HPLC on the C4 column.

The diverse inhibitory potency of the glycosaminoglycans on each of the three histones is consistent with the complex nature of the interaction, which cannot be explained by charge alone. If the action of the inhibitors on the haemagglutinating activity of the complex would result from their interaction with the individual histones, it would be expected that their minimum inhibitory concentration for the complex would be the average of those for the isolated histones. This is not the case; in fact, in many cases the haemagglutinating activity of the complex is sensitive to smaller concentrations of the inhibitors than that of the separated histones, showing that the interaction with the nucleic acid produces changes in their binding capacity.

As the heparin-binding specificity appears to belong to histone H4, this result suggests that the high molecular complex formed by the three histones and DNA, is bound to the affinity column by means of histone H4.

Heparin has also been reported to bind reversibly to intact core nucleosomes [26] which contain approximately 200 bp of DNA associated with a histone octamer that consists of two copies each of the core histones (H2A, H2B, H3 and H4) [27]. Upon heparin binding, the nucleosome assumes a new structure in which DNA is readily accessible to enzymes, such as DNase I and RNA polymerase I. Our

results are consistent with these findings, having characterized histone H4 as the responsible of the heparin-binding property. Not much is known about the changes that occur in the structure of chromatin fibre during transcription or replication and several mechanisms have been proposed. Our results support the hypothesis that the binding of nuclear proteoglycans to histones could be associated with these structural changes that occur in chromatin at transcription and replication.

On the other hand, several lines of evidence showed antiproliferative effects of heparin. These effects were observed on hepatoma cells [28, 29] as well as on vascular smooth muscle cells [30, 31], where the 3-O sulfate glucosamine in heparin would play a fundamental role [32]. Also, histone H2A.X was identified as a growth factor secreted by an androgen-independent subline of mouse mammary carcinoma cells [33]. These findings indicated that cell proliferation may be influenced by various heparin/haparan sulfate related glycosaminoglycans, with apparently widely differing structures, hence presumably by different mechanisms [1]. The results presented here suggest that one of these mechanisms could involve the interaction of these glycosaminoglycans with nuclear histones, thus modulating gene transcription.

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