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Heparin-Binding Lectin from Human Placenta: Purification and Partial Molecular Characterization and Its Relationship to Basic Fibroblast Growth Factors†

Beatrix Kohnke-Godt and Hans-Joachim Gabius*

Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, Hermann-Rein-Strasse 3, D-3400 Göttingen, Federal Republic of Germany

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ABSTRACT: The heparin-binding lectin from human placenta is isolated on the basis of its tendency to form large aggregates by gel filtration and on the basis of its affinity for heparin by affinity chromatography. The purified lectin dissociates into up to four distinct polypeptides with molecular weight values of 14400, 15000, 16200, and 16700 and a single isoelectric point of 9.0. Molecular heterogeneity is not due to different degrees of glycosylation, as evidenced by gel electrophoretic analysis after extensive treatment with various endoglycosidases. Despite its similarities of affinity to heparin, molecular size, and isoelectric point to the basic fibroblast growth factor (bFGF), the comparatively high yield of the lectin (approximately 1.5 mg/100 g of placenta), the occurrence of proteolytic fragmentation in the presence of heparin, and the lack of homology to the amino-terminal sequence of the lectin argue against any notable relationship to bFGF. Most importantly, the lack of mitogenic activity in a commonly used bioassay with quiescent 3T3 fibroblasts rules out any FGF-like activity on cell proliferation. The heparin-binding lectin is thus clearly distinguishable from heparin-binding growth factors. By employing biotinylated heparin as labeled ligand to visualize and quantify heparin binding, hapten inhibition in a solid-phase assay reveals that except for heparin no other vertebrate glycosaminoglycan but the sulfated fucan fucoidan can effectively reduce the Ca^{2+}-independent ligand binding. Proteolytic fragmentation by chymotrypsin in two independent assays demonstrates that a fragment of M_r 7800 still retains ability to bind heparin. The interaction of this lectin with naturally occurring heparin-like molecules may physiologically be involved in modulatory regulation of heparin-mediated processes.

The structure–function relationship of complex carbohydrates of cellular glycoconjugates has up to now not been elucidated to the extent of those of proteins. On the basis of the attractive assumption that functional and positional information can be stored, too, in a glycobiochemical code in complex carbohydrates, the specific interaction of carbohydrate sequences and proteins within diverse biological processes warrants special attention (Sharon, 1984; Schachter, 1984; Baenzinger, 1985; Gabius, 1988a; Rademacher et al., 1988). On the ligand side, the carbohydrate part of the ubiquitous proteoglycans, especially heparin/heparan sulfates, exhibits a prodigiously high degree of structural diversity, a presupposition for principally offering a great potential for functional involvement (Evered & Whelon, 1986; Gallagher et al., 1986). Deducing the organization and reactive interplay of regulatory events necessitates structural and functional characterization of the individual components.

Concerning the notable biological activities of the iduronic acid containing glycosaminoglycans, it is not surprising that a diverse array of proteins can bind to them, among them the heparin-binding growth factors. They comprise a family of proteins with pronounced effects on cell proliferation, cytodifferentiation, and morphogenesis of a wide variety of cells (Thomas & Gimenez-Gallego, 1986; Gospodarowicz, 1988; Lobb, 1988). Synonymously referred to as fibroblast growth factors (FGFs), they are generally classified into acidic and basic forms according to their isoelectric point. Moreover, they

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* To whom correspondence should be addressed.

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exist in several molecular forms within the range of 13–18 kDa that are products of proteolytic processing (Lobb et al., 1986; Moscatelli et al., 1988). Their common characteristics of strong affinity to heparin and, remarkably, their monomer size are shared by another class of proteins, recently discovered in chicken and rat liver and also in mammalian tumors (Ceri et al., 1981; Roberson et al., 1981; Gabius et al., 1984; Gabius, 1987a,b). Members of this group, the endogenous lectins, are also supposedly implicated in diverse biological recognition phenomena (Barondes, 1986; Gabius, 1988b, 1989). Accordingly, the appealing hypothesis has been put forward that the traced similarities between FGFs and lectins may reflect a functional relationship (Caron et al., 1988). Since biochemical studies to infer the molecular nature of the receptors, responsible for the histochemically detectable binding of heparin in tissue sections of the human placenta (Debbage et al., 1988), are in itself called for, we herein describe the characterization of the heparin-binding lectin from this abundant and readily available source of human tissue to likewise resolve the issue of the possible relationship between heparin-binding lectins and heparin-binding growth factors.

Materials and Methods

Materials. Cyanogen bromide was obtained from Merck (Darmstadt, FRG). Sepharose 4B, Sepharose CL-2B, and dextran sulfate-2.3 were from Pharmacia (Freiburg, FRG), and carrier ampholytes for isoelectric focusing were from Serva (Heidelberg, FRG). Heparin was purchased from Biomol (Ilvesheim, FRG) and together with heparan sulfate of bovine kidney from Sigma (Munich, FRG), the latter being mainly used for analytical purposes. Desulfation of commercially available heparin was achieved with dimethyl sulfoxide containing methanol (Inoue & Nagasawa, 1976). The individual monosaccharides, hyaluronic acid (from human umbilical cord), chondroitin 4-sulfate (chondroitin sulfate type A from whale cartilage), chondroitin 6-sulfate (chondroitin sulfate type C from shark cartilage), dermatan sulfate (chondroitin sulfate type B from porcine skin), the sulfated fucoidan, carrageenans (κ and λ), poly(galacturonic acid), deoxyribonucleic acid (degraded free acid from herring sperm), sperm nuclei (type II-S), protamine sulfate, histones (type VIII-S from calf thymus), micrococcal nuclease, and chymotrypsin were from Sigma (Munich, FRG). κ-Carrageenan is composed of carbohydrate chains of α-(1,3)-linked units of carrabiose, forming a linear alternating sequence of D-galactose 4-sulfate and 3,6-anhydro-D-galactose units, while λ-carrageenan carries three sulfate groups per disaccharide that is connected in α-(1,3) and α-(1,4) linkages (Thomson & Fowler, 1981). The endoglycosidases endo-β-*N*-acetylglucosaminidase F (endoglycosidase F, glycopeptidase F free) and endo-β-*N*-acetylglucosaminidase H (endoglycosidase H) were from Boehringer Mannheim (FRG); *O*-glycanase of *Diplococcus pneumoniae* and *N*-glycanase of *Flavobacterium meningosepticum* were from Genzyme (Boston). Nitrocellulose (NC) sheets (0.2 μm) were purchased from Schleicher & Schuell (Dassel, FRG). Streptavidin-colloidal gold solution AuroProbe–streptavidin (20 nm) and the IntenSE II silver enhancement kit were obtained from Janssen (Nettetal, FRG). All other reagents were commercially available analytical grade.

Preparation of the Heparin-Binding Lectin from Human Placenta. Six hundred grams of wet Sepharose 4B was activated with 12 g of cyanogen bromide and addition of 114 mL of a 1.5 M triethylamine solution for strong activation, as described (Kohn & Wilchek, 1982), and 9 g of heparin was added. Residual reactive groups were blocked with 1 M ethanolamine. All further steps, carried out essentially as described by Ceri et al. (1981) with modifications, were done at 4 °C. Routinely, 100 g of human full-term placenta was homogenized for 1 min in a Waring blender in 450 mL of 75 mM phosphate-buffered saline (PBS, pH 7.2) containing 4 mM β-mercaptoethanol, 2 mM EDTA, 0.5 M NaCl, 1 mM benzenesulfonyl fluoride, and 0.1 M lactose. The extract was centrifuged at 100000*g* for 1 h, and the delipidated supernatant was applied to a 1-L Sepharose CL-2B column, equilibrated with PBS containing 0.5 M NaCl and 4 mM β-mercaptoethanol, at a flow rate of 30 mL/h. Fractions with lectin activity, all in the excluded volume, as determined by blue dextran, were pooled (400 mL) and incubated for 12 h with 80 mL of heparin–Sepharose 4B batchwise in a sample rotator. The slurry was then poured into a column (3 × 20 cm). The resin was carefully washed with buffer (10 mM Tris-HCl, pH 8.6, containing 4 mM β-mercaptoethanol and 0.1 M NaCl) and then eluted stepwise by raising the salt concentration to 1 and 3 M in a total volume of 150 mL and finally by 100 mL of 100 mM Tris–glycine buffer, pH 3.0. All samples after salt elutions were concentrated by ultrafiltration using a membranous filter (Diaflo Ultrafiltration Model 50 with a YM-5 membrane) and dialyzed in tubing impermeable to molecules of molecular mass greater than 6 kDa first against a 10 mM and then a 5 mM Tris-HCl buffer (pH 7.2) containing 4 mM β-mercaptoethanol. Following lyophilization, protein was determined by the dye-binding assay, adapted for microtiter plates, with bovine serum albumin as standard (Redinbaugh & Campbell, 1985). The content of nucleic acid in these fractions was assessed spectrophotometrically (Warburg & Christian, 1942).

Gel Electrophoretic Procedures. Gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS) was performed on a 10% or 12.5% running gel with a 3% stacking gel (Laemmli, 1970). Two-dimensional gel electrophoresis and isoelectric focusing by nonequilibrium pH gel electrophoresis were carried out, as described (O'Farrell, 1974; O'Farrell et al., 1977). An optimized silver staining procedure that uses ethanol as a fixative instead of the more harmful methanol without affecting the high sensitivity was generally applied (Blum et al., 1987). Special care was taken to avoid contamination with skin proteins, especially keratins ranging from relative molecular masses of 65 to 68 kDa, in this highly sensitive method (Tasheva & Dessev, 1983; Ochs, 1983).

Hemagglutination Assays. Hemagglutination assays were done in microtiter V plates using 25-μL dilutions of protein (fractions during purification or protein after purification at a concentration starting from 0.2 μg/μL), 25 μL of 0.9% NaCl solution, 25 μL of 1% bovine serum albumin solution in 0.9% NaCl, and 25 μL of either 10% trypsinized, glutaraldehyde-fixed rabbit erythrocytes (type I cells) or 10% type I cells that were additionally ethanol-washed (type II cells) in phosphate-buffered saline (Nowak et al., 1976; Kobiler & Barondes, 1979). The competitive inhibitor of lectin activity was diluted in saline and replaced saline in the assay.

Conditions for Enzymatic Deglycosylation. The standard conditions for cleavage were applied for 25 h at 37 °C in the cases of endoglycosidase F and endoglycosidase H as well as *N*-glycanase and for 6 h at 37 °C for *O*-glycanase, as suggested by the commercial supplier. The reactions were terminated by the addition of sample buffer used for the preparation of specimens for electrophoresis in the presence of sodium dodecyl sulfate and heat treatment at 95 °C for 5 min. Prior to analysis, the samples were stored at −20 °C.

Protein Sequencing and Computerized Sequence Comparisons. After separation on a 12.5% preparative running

gel in SDS gel electrophoresis, the 14.4-kDa protein was electroeluted after staining/destaining and band excision from the gel at 100 V for 16 h at room temperature in 25 mM Tris-HCl/132 mM glycine buffer (pH 8.3) containing 0.05% SDS. Residual dye and ionic detergent were removed by solvent extraction (Konigsberg & Henderson, 1983). The amino-terminal sequence was determined by the USC Microchemical Core Laboratory for protein sequencing. Sequence comparisons, performed on a Sperry 1100/83 mainframe computer, within the protein sequence database of the National Biomedical Research Foundation were carried out by using a RELATE program essentially as described (Vehmeyer et al., 1988).

Heparin-Binding Assay. Spotting detection of heparin binding to the heparin-binding lectin, immobilized on nitrocellulose squares (1 cm × 1 cm), was based on the specific binding of biotinylated heparin, synthesized as previously described (Debbage et al., 1988), and the subsequent, highly sensitive detection of the label biotin by colloidal gold, coated with streptavidin. This assay thus advantageously requires no radioactive materials, as generally used in assays for heparin-binding proteins (Roberson et al., 1981; Hirose et al., 1986; Smith & Knauer, 1987). It was appropriately standardized and optimized to assess the ligand specificity. In detail, following spotting of the solution of the lectin (0.5 μg of protein), the nitrocellulose squares were allowed to dry for 15–30 min. The squares, placed in separate wells of tissue culture plates, were then taken through a series of steps, similar to immunospotting detection (Gabius et al., 1983). Extensive blocking of residual binding sites on the nitrocellulose was carried out for 30 min at 37 °C with a 5% solution of bovine serum albumin in 20 mM Tris-HCl buffer, pH 8.2, containing 0.9% NaCl, followed by thorough washing with the same buffer containing 0.1% bovine serum albumin and incubation with 20 μg/mL biotinylated heparin in the absence or presence of inhibitors in the same buffer for 1 h at room temperature. To remove unbound ligand, the nitrocellulose squares were washed with 1 mL of the above buffer for three rapid buffer changes and for three changes after 5 min. For quantification of the bound biotinylated heparin on the nitrocellulose, commercially available streptavidin–colloidal gold solution at a dilution of 1:100 in the above buffer, supplemented by the addition of 1:20 (v/v) dilution of gelatin, was applied for 2 h at room temperature. Enhancement of the staining reaction by signal amplification using a commercially available kit was performed according to the instructions of the manufacturer prior to evaluation by scanning, using a Desaga CD60 scanning device. Binding assays were also performed after treatment of the lectin (150 μg) with 20 μg of micrococcal nuclease in 100 mM Tris-HCl (pH 9.0) containing 10 mM $CaCl_2$ for 24 h at 4 °C, as described above.

Proteolytic Fragmentation of Heparin-Binding Lectin in the Absence or Presence of Heparin. Lyophilized lectin (100 μg) was dissolved in 30 μL of 0.5 M Tris-HCl (pH 7.7) and 6 M guanidine hydrochloride and then diluted 1:10 with 1% NH_4HCO_3, and limited proteolysis proceeded at an enzyme-to-substrate ratio of 1:30 (w/w) for 2 or 4 h at 37 °C. Enzymatic digestion was terminated by the addition of the protease inhibitors benzenesulfonyl fluoride and aprotinin, and the sample was electrophoretically analyzed. Another batch of lectin was incubated with heparin–Sepharose 4B for 12 h at 4 °C following proteolytic digestion at room temperature to identify heparin-binding fragments by affinity chromatography, as described above. Moreover, in situ cleavage under similar conditions was performed with 400 μg of lectin, immobilized to heparin on the affinity resin. A 10–18% linear gradient gel containing 7 M urea with an acrylamide:bis-(acrylamide) ratio of 20:1 that is effective to lower leakage of polypeptides from the matrix was used in the Laemmli discontinuous buffer system for gel electrophoretic analysis (Hashimoto et al., 1983).

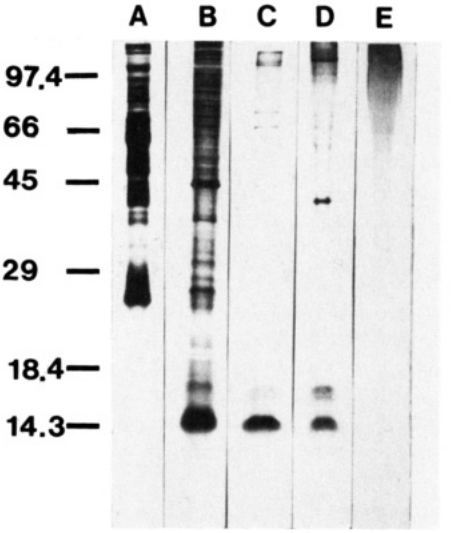

FIGURE 1: Visualization of the pattern of proteins by silver staining after reducing SDS gel electrophoresis on a 10% running gel in the supernatant after ultracentrifugation (4 μg, lane A), in the combined, activity-containing fractions after subsequent gel filtration on Sepharose CL-2B (4 μg, lane B), and in the fractions after elution from the heparin column with 1 M NaCl (1 μg, lane C), with 3 M NaCl (1 μg, lane D), and with 100 mM Tris/glycine buffer, pH 3.0 (lane E). Molecular mass markers, used in this and subsequent figures, are indicated by bars: phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.3 kDa).

Assay of Mitogenic Activity of the Heparin-Binding Lectin. Mouse 3T3 fibroblasts, used routinely as target cell type for assessing the activity of a growth factor from human placenta (Sen-Majumdar et al., 1986), were plated at a density of 5 × 10^3 or 10^4 cells per well, respectively, in 100 μL of RPMI 1640 medium containing 10% fetal calf serum, 2 mM glutamine, and 200 μg/mL penicillin/streptomycin. After 2 or 7 days, respectively, at 37 °C, the cells were incubated for 24 or 48 h with lectin concentrations ranging from 3 × 10^{-4} to 3 × 10^{-8} M prior to assessment of the cell number.

RESULTS

Purification and Characterization of the Heparin-Binding Lectin. To screen fractions from separation on columns for activity, advantage was taken of the ability of the heparin-binding lectin to agglutinate rabbit erythrocytes that besides successive treatments with trypsin as well as glutaraldehyde had been ethanol-washed, called type II erythrocytes (Kobiler & Barondes, 1979). Due to its tendency to appear as a highly aggregated form upon gel filtration, the lectin activity of human placenta was found in the void volume during gel filtration, drastically reducing the protein content and altering the polypeptide composition of the combined fractions relative to the extract (Figure 1, Table I). Subsequent affinity chromatography on heparin–Sepharose yielded two fractions of the heparin-binding lectin after salt elution at 1 and 3 M, respectively (Figure 1). Routinely starting from 100 g of human placenta, the first elution was found to amount to approximately 1.5 mg of protein, whereas the relative protein content of the second elution was only 5–8% of that of the first elution. Further elution attempts with Tris–glycine buffer (pH

Table I: Purification of the Heparin-Binding Lectin from Human Placenta[a]

purification step	total protein (mg)	sp act. [units/(mg·mL)]	purification (*x*-fold)
100000*g* supernatant	5000	10	1
Sepharose CL-2B	370	270	27
heparin–Sepharose (1 M eluate)	1.5	5000	500

[a] Lectin activity was determined in hemagglutination assays, inhibitable by heparin as competitive inhibitor, using glutaraldehyde-fixed, trypsin-treated rabbit erythrocytes that had additionally been washed with ethanol (type II erythrocytes), and the activity is expressed as 1/titer. The titer is the highest dilution of extract that agglutinated type II erythrocytes, as measured by serial 2-fold dilution. The specific activity is expressed as 1/titer divided by milligram of protein per milliliter of extract.

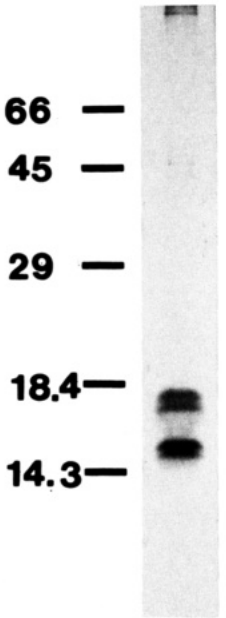

FIGURE 2: Visualization of the heparin-binding lectin (1 μg) following elution from the heparin column with 1 M NaCl by silver staining after reducing SDS gel electrophoresis on a 12.5% running gel.

3.0) did not result in any protein yield, excluding the presence of any tightly bound protein (Figure 1). The material from the two salt elution steps of different purifications consistently contained up to four different protein bands. Their relative proportions may differ from batch to batch, as already noted for a heparin-binding lectin from chicken liver (Ceri et al., 1981). The four individual bands had molecular weight values of 14400, 15000, 16200, and 16700 (±500), respectively, as determined in a 12.5% gel (Figure 2). Their molecular relationship is at present unclear. Besides the protein, spectrophotometric measurements of the purified lectin allowed detection of 8–10% (w/w) of nucleic acid in the different purified lectin fractions.

Only within the elution step of 3 M NaCl, two contaminating proteins could consistently be found that comigrated with actin and myosin (Figure 1). Each of the fractions was shown to meet the criterion for definition as lectin by hapten-inhibitable hemagglutination (Figure 3). Lectin amounts of as low as 5 ng per assay were able to cause agglutination of type II erythrocytes, competitively inhibited by the addition of heparin. Repeated cycles of freezing and thawing considerably reduced the lectin activity.

Further physical characterization was achieved by two-dimensional gel electrophoresis. The tendency of the lectin fraction to appear in a highly aggregated form was underscored

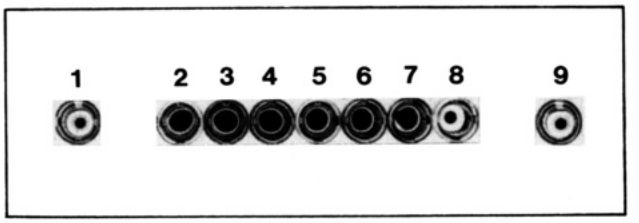

FIGURE 3: Hemagglutination of rabbit erythrocytes (type II cells) by the heparin-binding lectin. Well 1 shows a control without lectin addition, well 2 demonstrates the effect of 5 μg/assay lectin, and wells 3–8 contain lectin amounts of 2.5 μg, 500 ng, 50 ng, 25 ng, 5 ng, and 2.5 ng, respectively. Inhibition of hemagglutination by heparin (50 μg) is shown in the presence of 5 μg of lectin (well 9).

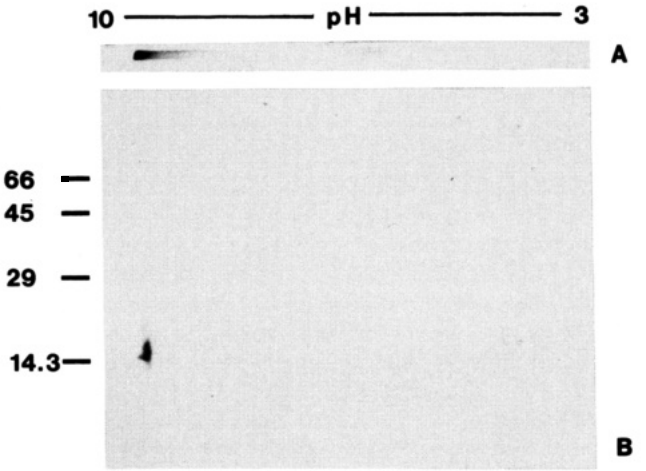

FIGURE 4: Visualization of the heparin-binding lectin by silver staining after two-dimensional gel electrophoresis involving isoelectric focusing in the first dimension and reducing SDS gel electrophoresis on a 12.5% running gel in the second dimension.

by the fact that the heparin-binding proteins, comprising the aggregates, only entered the gel in the presence of urea, when subjected to isoelectric focusing. Upon isoelectric focusing in the presence of urea in the first dimension and reducing SDS gel electrophoresis in the second dimension, a single p*I* of about 9.0 was seen for the proteins of the lectin fraction (Figure 4). Similarity in this property can be judged to indicate a close molecular relationship. The strongly basic p*I* value was independently confirmed by nonequilibrium pH gel electrophoresis, reversing the sample application relative to the gradient positions (not shown).

To probe whether carbohydrate modifications could be present on the individual polypeptides, the lectin underwent extensive treatment with different endoglycosidases. Neither endoglycosidase F or endoglycosidase H nor *N*-glycanase or *O*-glycanase was effective to alter the gel electrophoretic pattern, pointing to the absence of any carbohydrate modification that is accessible to glycosidases (not shown).

Amino-Terminal Sequence Analysis and Computerized Sequence Comparison. Since the basic p*I* value as well as the strong affinity to heparin suggested a possible relationship of the lectin to the basic fibroblast growth factor, we chose to determine the amino-terminal sequence of one individual protein in the lectin fraction. Since the ratio of the different proteins might well be influenced by proteolytic cleavage and since the smallest, in some batches, predominant protein band exhibited a relatively smaller molecular weight value than most forms of FGFs, its amino-terminal sequence should, if present, thus be found within the growth factor sequence. Electroelution yielded a homogeneous fraction of this polypeptide (Figure 5). Repetitive cycles of Edman degradation allowed us to derive the amino-terminal sequence from the major peaks

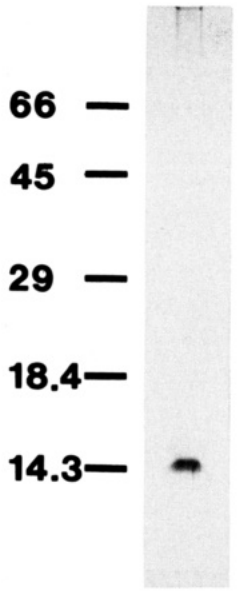

FIGURE 5: Visualization of the 14.4-kDa polypeptide following electroelution, as used in sequence analysis, by silver staining after reducing SDS gel electrophoresis on a 10% running gel.

Ala - Gln - Lys - Pro - Ala - Thr - Lys - Lys - Val -
Lys - Arg - Pro - Arg - Lys - Glu

FIGURE 6: Amino-terminal sequence of the first 15 positions of the 14.4-kDa polypeptide, derived from the major peaks of repetitive Edman degradation.

of the individual cycles (Figure 6). This sequence bore no relationship to any sequence published for a heparin-binding growth factor. Yet it revealed several basic amino acids that principally are required for heparin binding. Furthermore, any conceivable relationship to heparin-binding growth factors was definitely ruled out by two other criteria: First, the heparin-binding lectin had no significant mitogenic activity in a bioassay on quiescent 3T3 fibroblasts in the concentration range of 3×10^{-4} to 3×10^{-8} M. Thus, the lectin itself is not mitogenic, and it cannot be contaminated by FGF. Second, the lectin yield of approximately 1.5 mg, starting from 100 g of placenta, was considerably higher than the growth factor yield from the same type of organ (Gospodarowicz et al., 1985; Moscatelli et al., 1986). These two placental growth factors were reported to have molecular weight values of 16 000 and 18 700, respectively.

Computerized sequence comparisons, however, uncovered a notable relationship of the amino-terminal lectin sequence to sequence segments of histones. The summarized score of the individual comparisons, based on a mutation data matrix, was in the range of 32–45 for the 15 amino acid segment. Although this value is generally not considered to be remarkably significant, determination of the binding specificity in a solid-phase assay for heparin binding was nevertheless employed to deliberately exclude any functional relationship of the lectin to histones. In that case, histones should be able to compete for lectin-specific binding sites in the structure of heparin.

Binding Specificity of the Lectin. In order to facilitate quantitative determination of the ligand specificity of the heparin-binding lectin, we utilized biotinylated heparin as labeled ligand. Thereby, the drawbacks of special handling and the short half-life of radioactive tracers are overcome without any loss in sensitivity. This is due to the availability

Table II: Inhibition of Heparin Binding to the Immobilized Lectin in a Solid-Phase Assay

inhibitor	% inhibition for inhibitor:labeled heparin (w/w) ratio of 10:1	90:1	500:1
heparin	90	90	100
heparin (desulfated)	5	15	25
heparan sulfate	*b*		
dermatan sulfate			
chondroitin 4-sulfate			10
chondroitin 6-sulfate			
hyaluronic acid			
dextran sulfate-2.3	40	40	60
fucoidan	90	95	95
carrageenan-κ	20	40	60
carrageenan-λ	70	90	90
poly(galacturonic acid)		10	40
galacturonic acid		10	40
galacturonic acid 1-phosphate			
glucuronic acid			
sialic acid		30	60
fucose 1-phosphate		10	nd[a]
glucose 1-phosphate			
galactose 1-phosphate			
mannose 1-phosphate			
fructose 1,6-phosphate			
cellobiose			20
oligodeoxynucleotides	10	20	90
sperm nuclei	80	90	95
histones			
protamine sulfate			

[a] Not determined; further neutral sugars tested (fucose, *N*-acetylgalactosamine, and *N*-acetylglucosamine) were ineffective up to a 500-fold excess in relation to biotinylated heparin. [b] Blanks indicate that no inhibition was detected.

of signal enhancement reactions in the avidin–biotin system. Biotinylated heparin is the result of a chemical modification introducing an aliphatic linker for the biotin group. To be able to use it as a versatile probe for heparin binding, it was essential to show that the labeled ligand retained its ability to specifically interact with the lectin rather than to bind by simple ionic or hydrophobic interactions. Actually, binding of the labeled heparin to the lectin that had been immobilized onto nitrocellulose was effectively abolished by unmodified heparin. This inhibition verified the important feature of the solid-phase assay that immobilization appeared not to influence specific ligand recognition. Consequently, the quantitation of the extent of hapten inhibition could properly be assessed (Table II). Although removal of sulfate groups from heparin reduced its ligand capacity, the binding apparently was not mainly governed by charge interactions. Other glycosaminoglycans failed to inhibit the recognition of heparin by the immobilized lectin, whereas a sulfated fucan proved to be a potent inhibitor. Among the saccharides tested, only galacturonic acid, sialic acid, and cellobiose revealed some inhibitory potency. Other charge-carrying substances such as several phosphorylated sugars and glucuronic acid had no impact on ligand binding. Strong inhibition was seen with nucleic acids. Histones and protamine sulfate, however, failed to affect the binding. The sequence homology, noted in the computerized comparison, apparently did not reflect a similar behavior with respect to heparin binding. The presence of clusters of positive charges on different proteins and negative charges on a polysaccharide chain will thus not necessarily lead to identically effective binding.

The binding of heparin was not dependent on the presence of Ca^{2+} ions, because their withdrawal by chelating agent revealed no different results. Since the purified lectin still

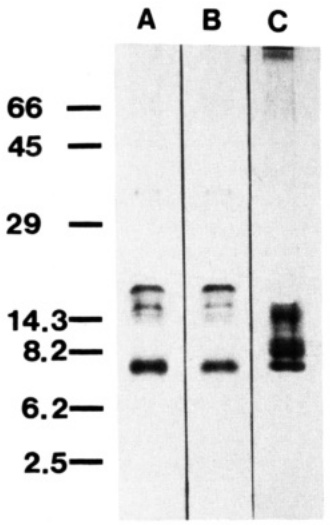

FIGURE 7: Separation and visualization of the pattern of polypeptides after chymotrypsin digestion of the heparin-binding lectin by reducing SDS gel electrophoresis in the presence of 7 M urea on a 10–18% linear gradient gel and by silver staining. The lectin was incubated for limited proteolytic cleavage with chymotrypsin in a 1:30 (w/w) ratio for 2 h at 37 °C, and the cleavage pattern was analyzed before (A) and after subsequent purification of heparin-binding polypeptides by affinity chromatography (B). Similar conditions were applied for cleavage of the lectin, immobilized on heparin–Sepharose, and heparin-binding fragments were isolated after extensive washing by salt elution from the affinity column (C). Additional molecular mass markers are fragments of myoglobin, designated by bars.

contained nucleic acids, extensive treatment with micrococcal nuclease was included to prove whether this association might influence the ligand binding. Immobilized lectin that had undergone extensive nuclease digestion, however, exhibited a rather similar extent, as reported earlier for the chicken lectin (Roberson et al., 1981), and specificity of binding. The only exception within the series of inhibition studies was the behavior of heparan sulfate. Its inhibitory capacity improved to a level of 25% of inhibition at an inhibitor:ligand ratio of 500:1 under these conditions (not shown).

Chymotrypsin Cleavage of the Heparin-Binding Lectin. The heparin-binding lectin was subjected to proteolytic cleavage in order to gain some insight into structural domains, relevant for ligand binding. The SDS gel profile after 2-h incubation with chymotrypsin in Figure 7A indicated primarily the appearance of one fragment of M_r 7800. Further incubation did not lead to increased fragmentation (not shown). Degradation of the lectin, followed by passage over a heparin–Sepharose column, allowed us to infer whether the resulting fragments will still retain affinity to heparin. Indeed, the pattern of polypeptides after affinity chromatography was identical (Figure 7B). Apparently, cleavage did not occur in the binding domain. It also could not impair any conformational characteristics, required for proper ligand binding.

An independent approach to identify heparin-binding domains used proteolytic fragmentation in the presence of heparin. Treatment of the basic fibroblast growth factor will, for example, not yield any fragments under these conditions (Baird et al., 1988). Changes in conformation and accessibility upon ligand binding may affect the fragmentation. In the case of the heparin-binding lectin, in situ cleavage on the affinity resin led to several fragments of a molecular weight range between the lowest protein band of the native lectin and the main fragment of proteolytic cleavage in the absence of ligand (Figure 7C). Sensitivity to cleavage was obviously enhanced for the upper protein bands of the lectin fraction. Conversion into smaller fragments, however, did not markedly reduce the affinity for heparin. Again, prolonging the incubation time to 4 h caused no change of the fragmentation pattern (not shown). Taken jointly, these results revealed that smaller fragments with an ability to bind heparin can be derived from the lectin. They are a step toward more clearly defining structural domains, responsible for specific ligand binding.

Discussion

The similarities in affinity to heparin and in molecular size and isoelectric point to truncated forms of the basic FGF notwithstanding, several characteristics strongly argue against a close relationship between FGFs and the heparin-binding lectin: comparatively high yield of the lectin from the same type of organ, proteolytic fragmentation in the presence of heparin, and lack of any homology in the determined partial lectin sequence. Most importantly, the lectin failed to exhibit mitogenic activity in a commonly used bioassay with quiescent 3T3 fibroblasts. This type of assay is nondiscriminatory in its response to a wide spectrum of growth factors. Whereas proteolytic processing is responsible for the various forms of basic FGF, the basis for the molecular heterogeneity of this lectin remains to be clarified. It is probably not due to different degress of glycosylation, because digestion with endoglycosidases did not eliminate the detected differences. Having established the basis to distinguish FGFs and the heparin-binding lectin, the question of its physiological function is consequently raised. The binding to nucleic acids, simplifying the purification, has already been considered artifactual, because only a small quantity of lectin is present in the nuclear fraction of chicken liver upon cell fractionation (Roberson et al., 1981). Notably, the lectin specifically interacts with heparin in the presence of nucleic acids. Histochemical evidence of nuclear localization of heparin receptors encourages thorough examination of this point in the human placenta (Debbage et al., 1988).

The ligand specificity of the placental lectin, exploited by hapten inhibition in a nonradioactive solid-phase assay, exhibits one marked difference in comparison to chicken and rat heparin-binding lectins, which are inhibitable by *N*-acetyl-D-galactosamine (Ceri et al., 1981; Roberson et al., 1981). It appears to be similar to a heparin-binding protein from bovine uteri with a molecular weight value of 78 000 and an isoelectric point of 5.2 (Lankes et al., 1988). This protein, apparently involved in inhibition of smooth muscle cell proliferation, also exhibited no substantial binding to other glycosaminoglycans besides heparin. Additionally, it discriminates to a varying degree against certain fractions of heparan sulfate of different origin, as also reported for inhibition of heparin binding to melanoma cells (Biswas, 1988). Incidentally, it is substantiated that the structural features of heparin that are less abundant in heparan sulfate are of key importance in mediation of attachment and outgrowth of implantation-stage blastocysts (Farach et al., 1987). Initial characterization of metabolically labeled proteoglycans from blastocyst-stage embryos prompted these authors to refer to heparin/heparan sulfate proteoglycans to indicate a certain extent of homology to heparin. Cloned aortic endothelial cells have likewise been reported to synthesize heparan sulfate proteoglycans, a small proportion of these molecules having the disaccharide structure of heparin (Nader et al., 1987). This clearly emphasized display of polymorphism within this class of proteoglycans calls for a thorough search for an endogenous ligand of the lectin that may have a restricted expression pattern (Ceri et al., 1979).

In this respect, it is interesting to note that several adhesive proteins, including laminin, thrombospondin, and von Willebrand factor, display a selective affinity for fucoidan. This

enables the sulfated fucan to be the most potent inhibitor of binding of sulfated glycolipids (Roberts & Ginsburg, 1988). Other exogenous sulfated polysaccharides like carrageenans have been found to specifically interact with cells of the mammalian immune system (Chong & Parish, 1986). They thus appear to resemble so far uncharacterized endogenous ligands in key structural aspects. In any case, it seems likely from the results of the ligand binding studies that the precise spatial orientation of the negative charge is more important than mere charge density per se. However, one plausible possibility should at present not be overlooked. Clusters of negative charges in a protein, acting analogously to heparin, may well prove to be a functional ligand. This has been exemplified for the binding of complement components to the heparin-binding domain of vitronectin (Tschopp et al., 1988). From the side of the protein, the lack of inhibition of binding of heparin to this lectin by histones that have a limited homology to the amino-terminal lectin sequence corroborates the conclusion that ligand binding is not simply governed by additive ionic interactions.

To further delineate the site for ligand binding of the heparin-binding lectin, two independent approaches have been employed. They consist of proteolytic cleavage in the absence of heparin and subsequent affinity chromatography as well as proteolytic cleavage in the presence of the ligand. Increased generation of fragments besides the main fragment at M_r 7800 and reduced resistance of the lectin itself to chymotryptic cleavage in the presence of heparin indicate differences in accessibility of cleavage points. The pattern of proteolytic fragmentation may also give clues for comparisons of the lectin to other known heparin-binding proteins. The pronounced presence of the main fragment of molecular weight of 7800 is theoretically compatible with the assumption of a potential relationship between the lectin and a recently described heparin-binding protein from macrophages within this molecular weight range (Wolpe et al., 1988). Its isoelectric point of 4.6, however, renders any close resemblance unlikely. Despite differences in molecular weight, a resemblance of the lectin and its proteolytic fragments to a heparin-binding protein from rat myoid cells, consisting of four distinct polypeptides with molecular weights of 10000, 13000, 13700, and 14600, cannot yet be eliminated (Kamo et al., 1986). Moreover, this is valid for a heparin-binding protein, detected by ligand blotting, in melanoma cell extracts that bears no immunologic cross-reactivity to FGF (Biswas, 1988).

In the physiological context, the multiplicity of proteins, specifically interacting with heparin-like sequences, presumably provides intricate possibilities for modulatory tuning. For the time being, we have to acknowledge that our information on the precise role of the heparin-binding lectin in this regulatory interplay is very limited indeed. Clarification of its function must await further experiments on the basis of the provided information.

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Secondary Structure of Charge Isomers of Myelin Basic Protein before and after Phosphorylation†

J. J. Ramwani,‡ R. M. Epand,§ and M. A. Moscarello*,‡

Research Institute, The Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada, and Department of Biochemistry, McMaster University, Hamilton, Ontario L8N 3Z5, Canada

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ABSTRACT: Human myelin basic protein (MBP) was fractionated into several of its charge isomers (components). Of these, the secondary structures of four isomers before and after phosphorylation have been studied by circular dichroism (CD). None of the four showed any α-helical structure. All of the components showed varying amounts of β-structure, random structure, and turns. Component 1 (C-1), the most cationic of the components, showed 13%; component 2 (C-2) had 19%; C-3, 17%; and C-4, 24% of β-structure. Each of the four components was phosphorylated with protein kinase C, from human brain. The extent of phosphorylation varied considerably from 2.8 ± 0.6 mol of PO_4/mol of protein in C-1 to 5.2 ± 0.8 mol of PO_4/mol of protein in C-4. The effect of phosphorylation on the secondary structure was to induce β-structure in all the components. The largest change in β-structure was in C-1 and the least in C-4. The surprising result is that although the components were phosphorylated to different extents, the amount of β-structure in all four components increased to a final proportion of 35-40%. Treatment of phosphorylated C-1 with acid phosphatase removed 50% of the total radioactivity. Although the remainder represented approximately 1 mol of PO_4/mol of protein, the proportion of β-structure was unaltered. We concluded that a single phosphorylation site identified as residues 5-13 represented a critical size for stabilization of β-structure of MBP in solution and that phosphorylation at the other sites had little influence on secondary structure.

Myelin basic protein (MBP) represents the major extrinsic protein of the myelin membrane, accounting for about 35% of the total protein responsible for the organization of the unique multilayered structure of myelin (Brady et al., 1981a; Epand, 1988). In model systems, the addition of MBP to vesicles composed of phosphatidylglycerol resulted in the formation of "crystalline" multilayers (Brady et al., 1981a). When compared to MBP isolated from myelin of victims of multiple sclerosis (Brady et al., 1981b), the latter was shown to be much less effective in organizing the formation of multilayers, implying that the MBP isolated from the MS material was less cationic than that isolated from normal brain. Since these observations could be explained by an increase in the relative proportions of the less cationic components, a detailed study of the charge microheterogeneity of MBP seemed appropriate.

Although purified MBP migrated as a single band on SDS-PAGE, on alkaline gels it could be resolved into 6-10 bands (components) on the basis of charge (Chou et al., 1976). These components were resolved on CM-52 columns at pH 10.6 as described originally by Chou et al. (1976). Since each component differed from the other by a single charge, we referred to them as charge isomers (Cheifetz & Moscarello, 1985). Thus, component 1, which was eluted last off the

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* To whom correspondence should be addressed.
‡ The Hospital for Sick Children.
§ McMaster University.

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