Heparin-Binding Lectin from Human Placenta: Further Characterization of Ligand Binding and Structural Properties and Its Relationship to Histones and Heparin-Binding Growth Factors

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ABSTRACT: We have previously demonstrated that the heparin-binding lectin of human placenta dissociates into up to four distinct polypeptides with molecular weights of 14400, 15000, 16200, and 16700 (Kohnke-Godt, B., & Gabius, H.-J. (1989) *Biochemistry 28*, 6531–6538). Stable complexes to ligands can shift the molecular weight appearance of the lectin to higher values. They can be dissociated in the additional presence of 9 M urea or by enzymatic degradation of heparin in model studies. The binding of heparin is rather stable over a range of salt concentrations from 1 to 3 M NaCl. Chemical modification with group-specific reagents to arginine, lysine, histidine, tyrosine, and tryptophan results in substantial inactivation of binding activity. Further amino-terminal sequence analyses point to a high-scoring relationship in this region to histone sequences, namely, histone H2B, but to no published sequences for any heparin-binding growth factor. Calculation of relatedness on the basis of differences in amino acid composition corroborates the conclusion of molecular distinction between the lectin, histones H2A and H2B, and the fibroblast growth factor as well as angiogenin. Histones only weakly agglutinate type II erythrocytes in contrast to the lectin. The immobilized lectin exhibits two classes of binding sites with K_D values of 3 and 110 nM in contrast to one estimated K_D value of 250 nM with a commercially available histone fraction. Both fractions retain binding activity to biotinylated heparin in transblots and are immunologically cross-reactive to antibodies, raised against the lectin as antigen. Subcellular fractionation clearly demonstrates that heparin-inhibitable hemagglutination activity and immunologically cross-reactive protein bands, characteristic for the lectin, but not unequivocally distinguishable from certain histone fractions in blots, are not confined to the nuclear fraction in the human placenta.

Heparin is known to be a heterogeneous mixture of highly charged glycosaminoglycans that can exert a variety of biological and biochemical effects (Jaques, 1979; Casu, 1985). Increasing attention is paid to its role in cellular processes like angiogenesis, cellular attachment, growth modulation, and smooth muscle cell proliferation (Hook et al., 1984; Folkman, 1985; Gallagher et al., 1986; Ruohslahti, 1989). The natural mast cell product is responsible for capillary endothelial cell migration in vitro (Azizkhan et al., 1980). Commercial heparin has proven to be a valuable tool to infer the conceivable role of a certain number of hitherto detected proteoglycans with heparin-like sequences, synthesized by other types of cells besides mast cells (Castellot et al., 1981; Farach et al., 1987; Nader et al., 1987). The widely documented specific binding of heparin to diverse types of cells persuasively underscores the possibility for its involvement to elicit subsequent biosignalling via interaction with cellular receptors (Kjellen et al., 1977, 1980; Glimelius et al., 1978; Busch et al., 1979; Carroll et al., 1982; Ryser et al., 1983; Handrow et al., 1984; Parish et al., 1984; Castellot et al., 1985; Thurn & Underhill, 1986; Bikfalvi et al., 1988; Biswas, 1988; Sobel & Adelman, 1988; Leung et al., 1989; Winer & Ax, 1989; Halper, 1990). Cell surface heparin-like structures can likewise serve as ligands for respective protein receptors, as exemplified by the dependence of adsorption of the pseudorabies virus to the presence of such carbohydrate sequences (Mettenleiter et al., 1990). Thus, the characterization of cellular receptors for heparin is a rational step in the quest to elucidate the biochemical basis of the specific binding and the heparin-mediated responses.

The strong affinity to heparin is shared by several classes of proteins including heparin-binding growth factors. Heparin-binding lectins, too, have been discovered in chicken and rat liver and also in mammalian tumors (Ceri et al., 1981; Roberson et al., 1981; Gabius et al., 1984; Gabius, 1987). Their presence is remarkable, because mammalian lectins are increasingly implicated in diverse regulatory processes, arguing in favor of the physiological relevance of recognitive protein (lectin)–carbohydrate interactions (Barondes, 1986; Gabius, 1988; Sharon & Lis, 1989). This reasoning has prompted the histochemical detection of appreciable amounts of heparin-binding sites and the hereby guided initial purification of the heparin-binding lectin from human placenta (Debbage et al., 1988; Kohnke-Godt & Gabius, 1989). For this report we have further investigated its subunit composition, ligand-binding properties, and structural features to delineate any relationship to other heparin-binding proteins. Neither histones nor any known heparin-binding growth factor convincingly displays the set of properties characteristic for the lectin, although histones exhibit pronounced similarities in amino-terminal sequences. Furthermore, subcellular fractionation reveals that hemagglutinating activity, immunologically cross-reactive to the lectin, is by no means confined to the nuclear fraction.

Materials and Methods

Materials. Cyanogen bromide was obtained from Merck (Darmstadt, FRG). Sepharose 4B, Sepharose CL-2B, and protein A–Sepharose 4B were from Pharmacia (Freiburg, FRG). Heparin was bought from Biomol (Hamburg, FRG) and also together with heparan sulfate of bovine kidney from

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Sigma (Munich, FRG), the latter being mainly used for analytical purposes. Histones (H 4255, type VII-S, comparable to H2B; H 6881, type VI-S, comparable to H2A; H 4380, type VIII-S, comparable to H3, as indicated by the commercial supplier—their appearance in gel electrophoretic analysis is documented in Figure 6) and avidin–peroxidase were from Sigma (Munich, FRG). Nitrocellulose sheets (0.2 μm) were purchased from Schleicher & Schuell (Dassel, FRG). Streptavidin–collodial gold solution (20 nm) and the IntenSE II silver enhancement kit were obtained from Janssen (Nettetal, FRG). Heparinase (E.C. 4.2.2.7) was obtained from ICN ImmunoBiologicals (Eschwege, FRG). 4-Chloro-1-naphthol, Tween-20, and horseradish peroxidase conjugated protein A were from Bio-Rad (Munich, FRG). [^{35}S]Heparin (3.7 MBq, 100 μCi) was from Amersham Buchler (Braunschweig, FRG). RNase A (E.C. 3.1.27.5) was purchased from Boehringer (Mannheim, FRG). Reagents for chemical modification were from Sigma (Munich, FRG). All other chemicals were commercially available analytical grade.

Preparation of the Heparin-Binding Lectin from Human Placenta. The isolation of the heparin-binding lectin from human placenta was carried out, as described in detail previously (Kohnke-Godt & Gabius, 1989). Instead of stepwise elution, the heparin-binding lectin was eluted from the heparin–Sepharose 4B column with a NaCl gradient, raising the salt concentration from 0.2 to 3 M NaCl. This experiment was performed at pH 7.2 in addition to the normally used pH value of 8.6 and in the presence of a variety of protease inhibitors throughout the complete processing to afford optimal protection against proteolytic degradation, namely, chymostatin, antipapain, leupeptin, benzamidine, and benzenesulfonyl fluoride. All samples after salt elutions were concentrated by ultrafiltration by 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 was performed on a 10% or 12.5% running gel with a 3% stacking gel (Laemmli, 1970). In specified cases 9 M urea was added to the sample buffer. 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 in relative molecular weights from 65K to 68K, in this highly sensitive method (Tasheva & Dessev, 1983; Ochs, 1983). To assess the influence of the ligand heparin on the electrophoretic mobility in the denaturing system, 10 μg of heparin was added to 2 μg of lectin. The ligand properties of heparin were destroyed by enzymatic digestion in parallel experiments. Two micrograms of lyophilized protein was dissolved in 15 μL of buffer containing 50 mM Tris-HCl, pH 7.0, 5 mM $CaCl_2$, and 0.15 M NaCl. Heparinase (0.01 unit) was added, and the sample was incubated at 30 °C for 5 h. The reaction was terminated by the addition of sample buffer, as used for the preparation of specimens for electrophoresis in the presence of sodium dodecyl sulfate, and by heat treatment at 95 °C for 5 min.

Heparin-Binding Assay. Spotting detection of heparin binding to the heparin-binding lectin, immobilized on nitrocellulose squares, 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, in conjunction with enhancement of the staining reaction and evaluation by scanning, as described in detail previously (Kohnke-Godt & Gabius, 1989).

pH and Salt Dependence of Heparin Binding. The pH dependence of heparin binding to the isolated lectin was characterized in the heparin-binding assay by changes of the buffer conditions. Lectin, 0.5 μg, was dissolved in various adjusted buffers containing 0.15 M NaCl–50 mM sodium borate (pH 10–8), 50 mM 3-(*N*-morpholino)propanesulfonic acid (pH 8–6), and 50 mM sodium acetate (pH 6–4). Alternatively, 20 mM sodium phosphate was adjusted to the appropriate pH values. The salt dependence of ligand binding to the isolated lectin (0.5 μg) was determined similarly in the heparin-binding assay by using 5 mM Tris-HCl buffer, pH 8.6, containing various concentrations of NaCl between 0 and 4 M.

Chemical Modification of Certain Groups of Amino Acid Residues. In each reaction 6.25 nmol of lectin was chemically modified by adding reagents that exhibit specificity to certain types of amino acid residues. To assess the degree of spatial protection by the ligand, the reagents were also incubated with lectin in the presence of 100 nmol of heparin. Modifications of amino groups with a 300-fold excess of citraconic anhydride at pH 8.0 and unblocking of the amino groups of the citraconylated lectin by incubation at 4 °C overnight at pH 3.0 were performed, as described (Dixon & Perham, 1968). ε-Amino groups of lysine residues were guanidinated in 0.2 M glycine/NaOH buffer, pH 10.5, by addition of an equal volume of 0.6 M *O*-methylisourea, adjusted to pH 10.5 with NaOH. The mixture was kept at 4 °C for 24 h. Arginine residues were modified with 50 mM cyclohexane-1,2-dione in 0.2 M sodium borate buffer at pH 9.0 and with phenylglyoxal in 20 mM phosphate-buffered saline, pH 8.0 (Patthy & Smith, 1975; Mukherji & Bhaduri, 1986). Tyrosine residues were modified with a 60-fold excess of *N*-acetylimidazole in 10 mM Tris-HCl buffer, pH 7.5, at 25 °C for 1 h (Riordan et al., 1965). Modification of tryptophan residues with *N*-bromosuccinimide at pH 4.0 was performed, as described (Spande & Witkop, 1967). Histidine residues were chemically altered by incubation with diethyl pyrocarbonate (Church et al., 1985). Glycine ethyl ester/ethyl[3-(dimethylamino)propyl]carbodiimide were used to modify carboxyl groups (Kundu et al., 1987). In all cases excess reagents were removed by dialysis against the reaction buffer at 4 °C prior to performing the solid-phase assay with biotinylated heparin as probe, outlined above and described in detail in our previous report (Kohnke-Godt & Gabius, 1989).

Protein Sequencing and Computerized Sequence Comparisons. After gel electrophoretic separation on a 12.5% preparative running gel, the four polypeptides were electroeluted after staining/destaining and band excision from the gels at 100 V for 16 h at room temperature in 25 mM Tris-HCl and 132 mM glycine buffer (pH 8.3) containing 0.05% sodium dodecyl sulfate. Residual dye and ionic detergent were removed by solvent extraction (Konigsberg & Henderson, 1983). The amino-terminal sequences were determined at 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 (Dayhoff, 1979). For determination of the amino acid composition, protein samples containing the four protein bands were thoroughly dialyzed against dilute acetic acid (0.5% v/v), freeze-dried, and hydrolyzed at 110 °C for 24 h in sealed tubes under nitrogen in 5.7 M HCl containing 0.23% phenol to protect tyrosine from oxidation and analyzed with a Durrum D-500 analyzer. An estimation of relationship between proteins was facilitated by calculating the $S\Delta Q$ values, derived from individual differences in mole percent of the amino acids according to Marchalonis and Weltman (1971).

Hemagglutination Assays. Hemagglutination assays were done in microtiter V plates by 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 1% bovine serum albumin (BSA) 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.

Ligand-Binding Studies. Nitrocellulose sheets were cut to pieces of radius 0.5 cm. Protein-containing solution was spotted on one piece in a total volume of 5 μL (1 μg of lectin or histone) and was allowed to dry on the nitrocellulose membrane. After blocking the residual protein-binding sites on the nitrocellulose for 30 min at 37 °C in 1 mL of buffer A (20 mM Tris-HCl, pH 8.2, containing 5% BSA and 0.9% NaCl) in 24-well culture plates, the nitrocellulose membranes were washed three times with buffer B (20 mM Tris-HCl, pH 8.2, containing 0.1% BSA and 0.9% NaCl). Different concentrations of [^{35}S]heparin were added in a total volume of 0.5 mL of buffer B and incubated for 75 min with the immobilized protein. Equilibrium was reached within this period, as ascertained in preceding studies. The concentration of the ligand ranged from 0.35 ng to 20 μg per assay (10 μg = 1.5 μCi). After they were washed three times with 1 mL of buffer B, the nitrocellulose membranes were dried, scintillation fluid was added, and the extent of ligand binding to the lectin or to the histone was assessed in a scintillation counter. Specific binding was referred to as total binding subtracted by the amount of nonspecific binding to the nitrocellulose membrane. The extent of nonspecific binding was found to be negligible; e.g., when using 1 μg of labeled heparin 60 cpm were measured as nonspecific, whereas 10900 cpm were the total binding. Transformation of the binding data for labeled heparin to obtain the dissociation constant K_D and to estimate the amount of binding at saturation was performed, as described (Scatchard, 1949).

Visualization of Heparin Binding after Electroblotting of Lectin and Histone. Transfer of lectin or histones (8 μg) after separation on a 10% running gel was carried out with the semidry blot system Sartoblott II (Sartorius, Göttingen, FRG). The two horizontal graphite plates enclosed a sandwich comprising (from the anodic plate) two layers of Whatman No. 1 filter paper soaked in 0.3 M Tris-HCl/20% v/v methanol (pH 10.4), one layer of filter paper soaked in 25 mM Tris-HCl/20% v/v methanol (pH 10.4), the nitrocellulose membrane as well as the polyacrylamide gel, both soaked in 25 mM Tris-HCl/20% v/v methanol (pH 10.4), and three layers of filter paper soaked in freshly prepared 40 mM 6-amino-*n*-hexanoic acid/25 mM Tris-HCl/20% v/v methanol (pH 9.4). Transfer was performed at room temperature for 15 min at a constant current of 400 mA. Thereafter, the blot was washed for 5 min in buffer A (20 mM Tris-HCl, pH 8.2, containing 0.9% NaCl and 1% BSA). Following blocking of residual protein-binding sites for 30 min at room temperature with buffer A that contained a total of 3% BSA and washing, the blot was subsequently incubated with heparin–X–biotin solution (0.2 mg/mL in buffer A) for 2 h at room temperature. After it was washed three times for 5 min with buffer A the blot was incubated for 1 h with avidin–peroxidase solution (10 μg/mL in buffer A). Color development was initiated by incubation of the membrane in a solution of 0.4 mg/mL 4-chloro-1-naphthol and 0.01% H_2O_2 in 20 mM Tris-HCl, pH 8.6.

Preparation of the Antibody. To raise specific polyclonal antibodies in rabbits, the lectin was transferred after electrophoretic separation in the presence of sodium dodecyl sulfate from polyacrylamide gels onto nitrocellulose membranes and stained with red Ponceau solution to visualize the proteins, as described for a β-galactoside-specific lectin (Bardosi et al., 1989). The centers of the lanes were carefully excized and destained in water. These protein-carrying nitrocellulose strips were minced with a mortar and pestle under liquid nitrogen and the resulting nitrocellulose powder containing approximately 80 μg of immobilized lectin in 1 mL of phosphate-buffered saline was injected intradermally without addition of Freund's adjuvant and without solubilization of the nitrocellulose with dimethyl sulfoxide (Diano et al., 1987). At 3-week intervals, three booster injections of 80 μg of protein on nitrocellulose were given, and blood was taken 1 week thereafter. The IgG fraction of serum was purified by chromatography on protein A–Sepharose 4B. Ouchterlony double diffusion, immunoblotting with placenta extract, and immunospotting with the indicator horseradish peroxidase conjugated protein A for determining antibody specificity were performed with the IgG fraction from preimmune serum and serum after injections, as described (Gabius et al., 1983). For Western blot analysis, proteins were electrophoretically separated and subsequently transferred to nitrocellulose, as described (Towbin et al., 1979). After transfer, nonspecific binding was blocked by 3 × 5 min incubation of the nitrocellulose in buffer A (1.59 mM KH_2PO_4, 8.4 mM Na_2HPO_4, 2.68 mM KCl, 137 mM NaCl, and 0.05% Tween-20). The nitrocellulose was then incubated with a 1:50 dilution of the antibody fraction against the heparin-binding lectin for 12 h in buffer A. After it was washed with buffer A the nitrocellulose was incubated for 90 min with a solution of horseradish peroxidase conjugated protein A (diluted 1:2000 in buffer A). The immobilized peroxidase conjugate was visualized by incubation with 0.01% hydrogen peroxide and 0.5 mg/mL 4-chloro-1-naphthol in buffer A containing 16% methanol but no Tween-20. Between the incubation steps the nitrocellulose membrane was washed three times with buffer A.

Subcellular Fractionation and Correlation of Lectin Activity to the Different Fractions. All steps were routinely performed at 4 °C. Fresh human placenta was washed with 8 volumes of 0.3 M sucrose, 4 mM β-mercaptoethanol, and 3 mM $MgCl_2$. The placenta was homogenized in 5 volumes of the same solution in a Waring blender for 15 s at low speed. The extract was filtered through Nytex and centrifuged at 12000*g* for 10 min. The supernatant was called the cytoplasmic supernatant fraction according to Roberson et al. (1981). The pellet was resuspended in 2 M sucrose, 4 mM β-mercaptoethanol, and 1 mM $MgCl_2$. To separate nuclei from other particulate material, a 5-mL aliquot of this suspension was layered over

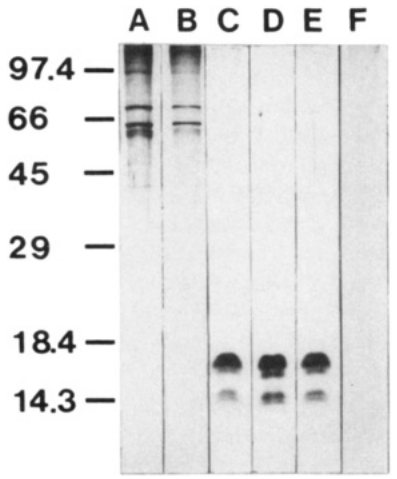

FIGURE 1: Visualization of the pattern of protein bands at different gradient positions, namely, at 0.5 M NaCl (lane A), 0.7 M NaCl (lane B), 1.0 M NaCl (lane C), 1.3 M NaCl (lane D), 1.6 M NaCl (lane E), and 2 M NaCl (lane F), within elution with increasing NaCl concentration from a heparin–Sepharose 4B column by a salt gradient from 0.2 to 3 M NaCl. Reducing sodium dodecyl sulfate (SDS) gel electrophoresis on a 12.5% running gel was followed by silver staining. 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), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.3 kDa).

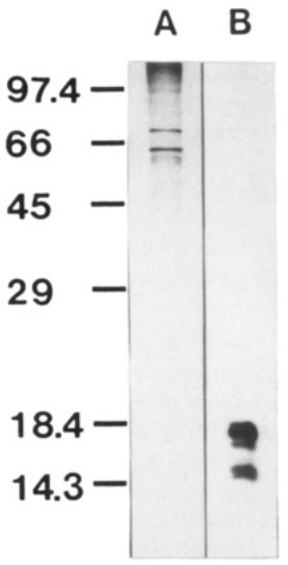

FIGURE 2: Influence of urea on the pattern of the heparin-binding proteins, obtained by elution from the heparin–Sepharose column with 0.7 M NaCl and visualized by silver staining after reducing SDS gel electrophoresis on a 12.5% running gel. Specimens from the gradient elution at 0.7 M NaCl were treated prior to gel electrophoresis with sample buffer in the presence of 3% SDS and 5% β-mercaptoethanol (1 μg of protein, lane A) and with sample buffer containing 9 M urea in addition to 3% SDS and 5% β-mercaptoethanol (2 μg of protein, lane B).

8 mL of 2 M sucrose containing 4 mM β-mercaptoethanol and 1 mM $MgCl_2$ and centrifuged at 40000g for 1 h in a swinging-bucket rotor. The top 3 mL were collected, termed the cytoplasmic particulate fraction, and the pellet is referred to as the nuclear fraction in accordance to Roberson et al. (1981). The nuclear fraction was homogenized with a Potter–Elvehjem homogenizer in 25 mM Tris-HCl buffer (pH 7.8) containing 200 mM NaCl, 0.1 mM benzenesulfonyl fluoride, 0.1% sodium deoxycholate, 1% Triton X-100, and 1 mM dithiothreitol. After centrifugation at 12000g, a DNA-rich pellet and a nuclear supernatant were obtained. For assessment of lectin activity by hemagglutination assays, aliquots of the particulate fractions were always carefully homogenized and diluted in phosphate-buffered saline, pH 7.2, to yield similar concentrations for promptly performed assays, as described above. To determine the DNA content of the subcellular fractions, total nucleic acid extracts were prepared with phenol/chloroform/isoamyl alcohol (25:24:1) (Marmur, 1961). DNA was distinguished from RNA by degradation of samples with RNase A prior to a new extraction with phenol/chloroform/isoamyl alcohol. The content of DNA in the individual fractions was measured spectrophotometrically (Warburg & Christian, 1942).

Results

Further Studies on Lectin Purification. The purified lectin had been shown to dissociate into up to four distinct polypeptides that exhibited a single isoelectric point of 9.0 (Kohnke-Godt & Gabius, 1989). Gradient elution with increasing salt concentrations was chosen to address the question, whether any further separation could be achieved within affinity chromatography. The four typical bands were found to invariably coelute at NaCl concentrations between 0.9 and 1.6 M in the gradient (Figure 1). No apparent separation among the four polypeptides was discernible. The pattern was maintained when each step within the purification was performed in the presence of a variety of protease inhibitors. Furthermore, gel electrophoretic analysis in the absence or presence of β-mercaptoethanol with or without boiling confirmed that this pattern was not generated by the sample preparation. When the pH value of the buffer in affinity chromatography was lowered from 8.6 to 7.2, the lectin activity eluted between 0.35 and 1.0 M NaCl.

Since the lectin had already been separated from other proteins due to its tendency to form high molecular weight aggregates in preceding gel filtration, the fractions with apparently lower affinity to heparin–Sepharose but with the capacity to form aggregates deserved further attention. They display increases in the molecular weight of the proteins (Figure 1). Specimens from gradient elution at 0.7 M NaCl concentration were examined, whether the detectable higher molecular weight of the protein bands was due to a different nature of the proteins or due to a residual complex formation of lectin to an anionic ligand in the extract. This maintenance of complex stability might explain the reduced affinity to the immobilized affinity ligand. Indeed, dissociation of the complexes in these fractions into the typical bands was obtained by adding 9 M urea to the sample buffer (Figure 2). Remarkably, this complex formation was thus resistant to the presence of sodium dodecyl sulfate and heating alone. To independently corroborate this conclusion, an excess of heparin was added to lectin fractions prior to electrophoretic analysis. Consequently, the typical lectin bands between molecular weight values of 14000 and 17000 disappeared in the presence of heparin. They were only discerned after ligand degradation by heparinase, emphasizing the stability of lectin–ligand complexes even under formally denaturing conditions (Figure 3). Association to ligands in solution under the conditions of extraction thus can cause marked variations in the profile within gel electrophoresis.

Dependence of Heparin Binding to the Lectin on Ionic Strength and pH. The heparin-binding activity of the lectin was characterized in a solid-phase assay over a range of salt concentrations from 0 to 4 M NaCl. A broad plateau of optimal binding activity was determined between 1 and 3 M salt concentration. In the absence of NaCl only 20% of the optimal binding activity was measurable. This finding sug-

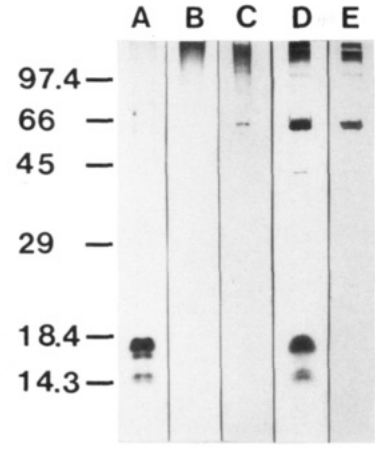

FIGURE 3: Influence of addition of heparin and heparinase on the pattern of the heparin-binding lectin after reducing SDS gel electrophoresis on a 10% running gel. One microgram of lectin was analyzed in the absence of additional heparin (lane A), 2 μg of lectin was incubated prior to electrophoresis with 10 μg of heparin (lane B), 2 μg of lectin was incubated prior to electrophoresis with 10 μg of heparin and sample buffer containing 9 M urea in addition to 3% SDS and 5% β-mercaptoethanol (lane C), and 2 μg of lectin was incubated with 10 μg of heparin in the presence of 0.01 unit of heparinase (lane D). To unequivocally assess the position of the enzyme in the analysis, an identical amount of heparinase was run on the gel (lane E).

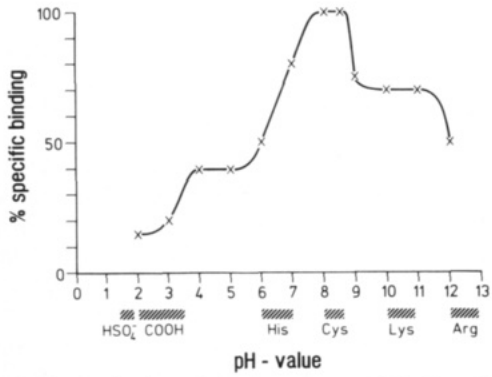

FIGURE 4: Determination of pH dependence of binding of heparin to the lectin. Heparin binding was determined in a solid-phase assay in phosphate buffer of various pH values. pK_a values of the common amino acid side chains and of HSO_4^- groups are given.

gested that heparin binding to the lectin is not mainly governed by ionic interactions that should be disrupted at higher salt concentrations. Other forces such as hydrogen bonds might contribute to the stability of complex formation. To raise evidence for the involvement of certain groups of amino acid residues in a first approach, the binding activity was assessed over a pH range that covered pK_a values of various types of amino acids. This experimental series will necessarily allow only a tentative correlation. When the pH value was altered stepwise over the range pH 1–12, increases were noted between pH 3 and 4 as well as between pH 6 and 8 (Figure 4). These observations phenomenologically implicated carboxyl groups on the protein and/or sulfate groups on the ligand as well as histidine residues in mediating the enhancement, because the pK_a values of the respective residues are located at these points. Losses of binding occurred between pH values of 8 and 9 and above 11, where ε-amino groups of lysine are mainly deprotonated. Remarkably, these activity alterations were independent of the nature of the buffer substance used. The higher measureable binding activity at pH 8.6 compared to that at pH 7.2 was in agreement to the increased affinity of the lectin to heparin–Sepharose, as determined by gradient elution with

Table I: Effect of Amino Acid Modification on Binding of Heparin to the Lectin in a Solid-Phase Assay

chemical treatment	residues modified	% inhib caused by modificatn	% inhib caused by modificatn in the presence of 100 nmol of heparin
none (native protein)		no inhib	
O-methylisourea	lysine	20	no inhib
citraconic anhydride	lysine, NH_2-terminal amino group	90[a]	40
cyclohexane-1,2-dione	arginine	70	no inhib
phenylglyoxal	arginine	60	70
N-acetylimidazole	tyrosine	80	40
N-bromosuccinimide	tryptophan	100	40
diethyl pyrocarbonate	histidine	100	90
ester–carbodiimide	carboxyl groups	no inhib	20

[a] Decitraconylation of the modified lectin led to a substantial recovery (20% inhibition).

NaCl at these two pH values. It should not be neglected that activity changes at nonphysiological pH conditions can well be caused by structural changes of the immobilized lectin. In order to independently infer the participation of certain amino acid side chains, chemical modification with group-specific reagents was employed.

Effect of Chemical Modification on Lectin Activity. To further characterize the nature of the lectin–heparin interaction, the effects that chemical modification exert on the binding were examined with several group-specific reagents. The inherent problem, whether a residue directly participates in ligand binding or is responsible to stabilize an active conformation, was experimentally taken into account by performing modification in the absence and presence of the ligand that is supposed to spatially protect crucial residues. Impairment of binding activity was evaluated in the solid-phase assay. The results are summarized in Table I. Modification of lysine residues with citraconic anhydride nearly completely inactivated the lectin. Decitraconylation of the modified lectin led to a partial recovery, excluding irreversible inactivation by the conditions of chemical modification. *O*-Methylisourea was comparatively less effective. The presence of the ligand moderately protected the lectin from disruption of binding activity, caused by lysine modification. Cyclohexane-1,2-dione and phenylglyoxal, modifying arginine residues, were equally effective to impair lectin activity. However, only access of the bulkier reagent cyclohexane-1,2-dione was restricted in the presence of heparin. Partial protection by heparin was measured in the cases of tyrosine and tryptophan modification that strongly reduced the activity. Incubation of the lectin with diethyl pyrocarbonate completely inactivated the lectin. This result is in agreement with the increase in binding activity when the pH value of the medium exceeded the pK_a value of histidine, pointing to a nonionic involvement in ligand binding. The presence of heparin only slightly reduced the inhibition. Overall, these experiments in the absence and presence of heparin revealed that the integrity of lysine and arginine residues as well as of the hydrophobic residues tyrosine and tryptophan and also histidine as potential donors for hydrogen bonding is of importance to retain binding capacity.

Amino-Terminal Sequence Analysis and Computerized Sequence Comparison. To unravel any structural relationship of lectin polypeptides to other heparin-binding proteins, amino-terminal sequence analysis was continued for the polypeptides beyond the respective measurement on the smallest polypeptide by repetitive cycles of Edman degradation, already reported (Kohnke-Godt & Gabius, 1989). In this case, the

Table II: Amino Acid Composition of Heparin-Binding Lectin, Basic Fibroblast Growth Factors (EGF) (Human and Bovine), Angiogenin, and Histones H2A.1, H2A.2, and H2B (Residues/Molecule)

amino acid	lectin	FGF		angiogenin	histone H2A.1	histone H2A.2	histone H2B
		human[a]	bovine[b]	(human)[c]	(human)[d]	(human)[d]	(human)[e]
Asp	7.2	12.0	11	15.96	8	9	6
Thr	6.3	6.0	4	7.35	5	4	8
Ser	5.8	8.3	9	9.80	4	5	13
Glu	9.8	13.1	13	11.47	12	11	10
Pro	4.2	nd	10	7.93	5	5	6
Gly	14.2	11.8	15	10.41	14	14	7
Ala	13.3	8.5	9	6.05	17	17	16
Cys	nd	nd	7	6.08	0	0	0
Val	6.9	5.9	5	4.86	8	8	8
Met	1.34	1.8	2	0.97	1	0	3
Ile	5.0	3.9	3	6.31	6	6	7
Leu	9.9	13.3	12	6.10	16	15	6
Tyr	2.8	5.7	6	3.99	3	3	5
Phe	2.2	6.7	7	4.90	1	1	2
His	2.4	2.8	2	5.72	4	4	4
Trp	nd	0.9	1	1	0	0	0
Lys	11.8	14.0	13	7.49	13	14	18
Arg	12.2	9.4	11	13.0	13	12	6

[a] Amino acid composition for human placenta FGF based on 124 amino acids per molecule according to Gospodarowicz et al. (1985). [b] Amino acid composition for bovine pituitary FGF based on 140 amino acids per molecule according to Böhlen et al. (1984). [c] Amino acid composition for human tumor-derived angiogenin based on 129 amino acids per molecule according to Fett et al. (1985). [d] Amino acid compositions of histones H2A.1 and H2A.2 were calculated on the basis of their published sequences (Hatch & Bonner, 1988; West & Bonner, 1983). [e] Amino acid composition of histone H2B was calculated on the basis of its published sequence (Zhong et al., 1983); nd = not determined.

minor peaks of the individual cycles allowed the derivation of another sequence. This sequence and further amino-terminal sequences together with the assignment of the polypeptide bands after gel electrophoresis of the sequences are documented in Figure 5. Interestingly, computerized sequence comparison uncovered an obvious relationship to histones. The amino-terminal sequence of polypeptide A is distinguished from the sequence of human histone H1b from residue 6 on or from the amino-terminal sequence of human histone H3 by three mismatches. Sequence B is identical with the amino-terminal sequence of human histone H2B. Similarly, the minor sequence of polypeptide D is identical with a stretch of amino acid residues 21–35 of human histone H2B, whereas the main sequence of polypeptide D contained five mismatches. No apparent relationship was noted to any heparin-binding growth factor in accordance to our previous report (Kohnke-Godt & Gabius, 1989).

To further assess the extent of the relationship between the lectin and histones or heparin-binding growth factors the amino acid composition of the lectin was determined. Compilation of amino acid compositions of other proteins, namely, three heparin-binding growth factors and three histones, indicated a relationship, but not an identity, between the lectin and histones (Table II). The sequence identities to histone H2B notwithstanding, the two amino acid compositions displayed significant differences. It should also be pointed out that other high-scoring sequence similarities had been unraveled, e.g., between the D_{main} sequence and the NH_2-terminal sequence of myosin L1 catalytic light chain.

A convenient method for estimation of the relationship from differences in amino acid compositions had been provided by Marchalonis and Weltman (1971). The individual differences in mole percent of the amino acids are squared and summed, yielding the so-called $S\Delta Q$ value, which is a measure of extent of sequence dissimilarity within protein families. When these calculations were carried out for the lectin, for histones, and selected growth factors, the range of values underscored a relationship but no identity of histones and, less markedly, growth factors to the lectin (Table III). Among these histones, the H2B type could be judged to be less related on the basis of the $S\Delta Q$ value compared to the H2A types despite its sequence identities. Nonetheless, complete sequence analysis is needed to resolve this question.

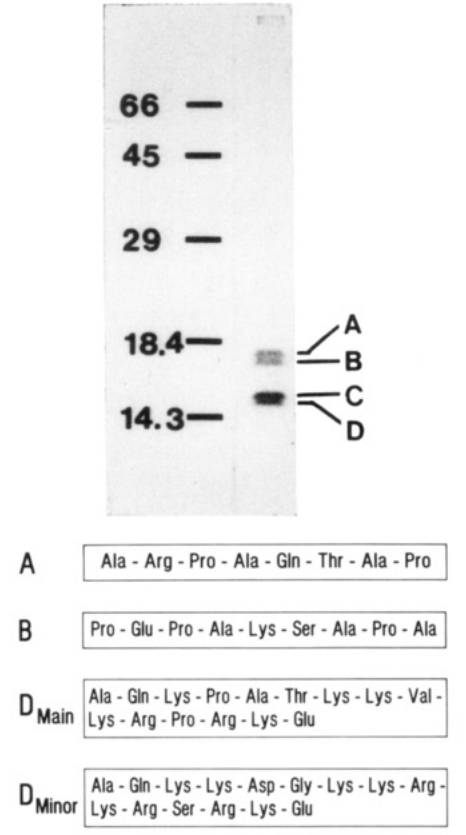

FIGURE 5: (A, Top) Polypeptide pattern of the heparin-binding lectin (1 μg) after silver staining and reducing SDS gel electrophoresis on a 12.5% running gel. (B, Bottom) Amino-terminal sequences of the polypeptides of the heparin-binding lectin, referred to by capital letters in part A.

Among the commercially available histones the H2B fraction showed a high degree of similarity in comparison to the lectin in the gel electrophoretic profile (Figure 6). Upon two-dimensional gel electrophoretic analysis, these fractions invariably displayed an isoelectric point of approximately 9.0,

Table III: Calculation of Relationship between Heparin-Binding Lectin, Basic Fibroblast Growth Factors (Human and Bovine), Angiogenin, and Histones H2A.1, H2A.2, and H2B, As Judged by Comparison of $S\Delta Q$ Values, Evaluated from Differences in the Amino Acid Compositions[a]

		FGF		angiogenin	histone H2A.1	histone H2A.2	histone H2B
	lectin	human	bovine	(human)	(human)	(human)	(human)
lectin	0	92	87	172	29	30	150
FGF (human	92	0	17	106	121	113	182
FGF (bovine)	87	17	0	105	126	118	205
angiogenin (human)	172	106	105	0	243	234	269
histone H2A.1 (human)	29	121	126	243	0	4.5	196
histone H2A.2 (human)	30	113	118	234	4.5	0	176
histone H2B (human)	150	182	205	269	196	176	0

[a] $S\Delta Q$ values were determined according to Marchalonis and Weltman (1971).

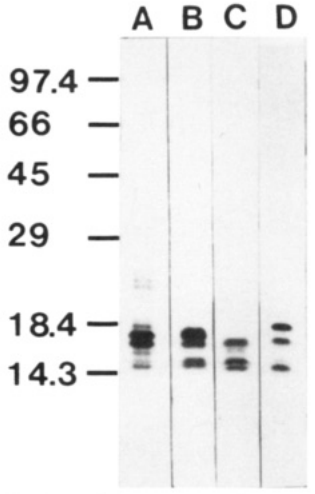

FIGURE 6: Visualization of the pattern of protein bands by silver staining after reducing SDS gel electrophoresis on a 12.5% running gel in the commercially available histone fraction type VII-S, equivalent to H2B (1 μg, lane A), in the heparin-binding lectin fraction (1 μg, lane B), in the histone fraction type VI-S, equivalent to H2A (1 μg, lane C), and in the histone fraction type VIII-S, equivalent to H3 (1 μg, lane D).

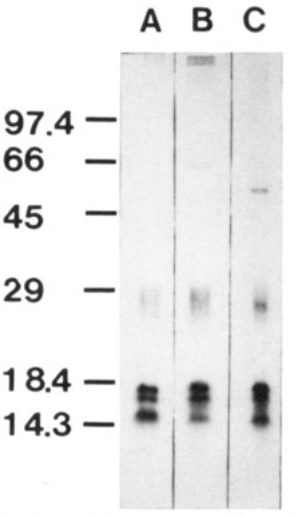

FIGURE 7: Visualization of the pattern of protein bands by silver staining after reducing SDS gel electrophoresis on a 10% running gel in the fractions after elution of histone type VII-S (equivalent to H2B) from the heparin–Sepharose column with 1 M NaCl (1 μg, lane A), with 3 M NaCl (1 μg, lane B), and with 6 M guanidine hydrochloride (1 μg, lane C). One milligram of histone (type VII-S) was dissolved in 50 mL of buffer A (10 mM Tris-HCl, pH 8.6, containing 4 mM β-mercaptoethanol and 0.1 M NaCl) and incubated batchwise for 12 h with 50 mL of heparin–Sepharose 4B in a sample rotator. The slurry was then poured into a column (1 × 10 cm). The resin was carefully washed with buffer A and then eluted stepwise by 150 mL of the specified solutions.

indistinguishable from that of lectin (not shown). The affinity to heparin, not only to DNA, was ascertained for the histone fraction, equivalent to histone H2B, by performing affinity chromatography under identical conditions, resulting in salt elution primarily at 1 M NaCl, not 3 M NaCl, and then with 6 M guanidinium hydrochloride from the column (Figure 7). When placenta extract was fractionated by the standard protocol, no characteristic bands could be visualized after elution with 6 M guanidinium hydrochloride. Therefore, the elution profiles of histone fractions and of the lectin in the fractionation scheme apparently were not identical. Nonetheless, since histones and the lectin in principle shared the affinity to heparin and certain structural elements, further comparative analysis of ligand-binding properties was warranted to unequivocally pinpoint discriminatory features.

Ligand Binding to Lectin and Histones. Since the potency to agglutinate erythrocytes is recognized as a characteristic property of lectins, this easily measurable parameter was monitored. Histones only weakly agglutinated type II erythrocytes. As holds true for the strong activity of the lectin, the comparatively weak agglutination of type II erythrocytes by histones was inhibitable by heparin. In the solid-phase assay sulfated polysaccharides like dermatan sulfate or carrageenans exhibited an increased inhibitory capacity to heparin binding to histones in relation to the lectin. Quantitation of binding properties was facilitated by extending the solid-phase assay using ^{35}S-labeled heparin as a ligand for the immobilized proteins. The binding was saturable and transformation of the binding data at equilibrium for the lectin revealed two classes of binding with apparent dissociation constants K_D of 3 and 110 nM (Figure 8A). A parallel study with the histone H2B yielded a set of points, where a plot could be less easily generated (Figure 8B). An approximation resulted in a single slope with a K_D value of 250 nM. Binding of heparin to histones at saturation was more than 3-fold higher than that measured with the lectin. Both fractions bound to heparin, although binding parameters and inhibition by sulfated polysaccharides differed. It was thus conceivable that they could be detected in transblots by labeled heparin. Generally, this approach can readily detect ligand-binding proteins in extracts, if they retain at least a measurable amount of activity. Indeed, after electrophoretic separation in the presence of sodium dodecyl sulfate and transfer to nitrocellulose membranes, the lectin and a histone fraction bound heparin, enabling their detection on blots (Figure 9). This observation has an important bearing on the interpretation of results obtained with this technique on extracts.

Immunological Cross-Reaction between Lectins and Histones. To provide a tool for lectin quantification and localization, polyclonal antibodies were raised to the lectin and immobilized on nitrocellulose after its isolation and its electrophoretic transfer to nitrocellulose. As could be expected on the basis of their dissociation into typical lectin components, illustrated in Figure 2, complexes were found to be cross-reactive in addition to the lectin bands (Figure 10). The extract

Table IV: Subcellular Localization of Lectin Activity in Human Placenta[a]

sample	vol (mL)	total protein (mg)	specific act. titer^{-1}/(mg of protein/mL of extract)		total lectin activity (titer^{-1} × mL of extract)		% total DNA
			GRTE[b]	EGTRE[c]	GTRE	EGTRE	
whole cell extract	315	1500	4	12	2010	23000	100
cytoplasmic supernatant fraction	310	1360	0.9	7.2	1240	9920	0
cytoplasmic particulate fraction	17	76	0.88	111	68	8500	8
nuclear fraction	10	15	100	333	500	7500	92

[a] Lectin activity was determined in hemagglutination assays with different types of erythrocytes. [b] Glutaraldehyde-fixed, trypsin-treated rabbit erythrocytes (type I erythrocytes). [c] GTR erythrocytes, washed with ethanol (type II erythrocytes). The activity is expressed as titer^{-1}. The titer is the highest dilution of extract that agglutinated erythrocytes, as measured by serial 2-fold dilutions.

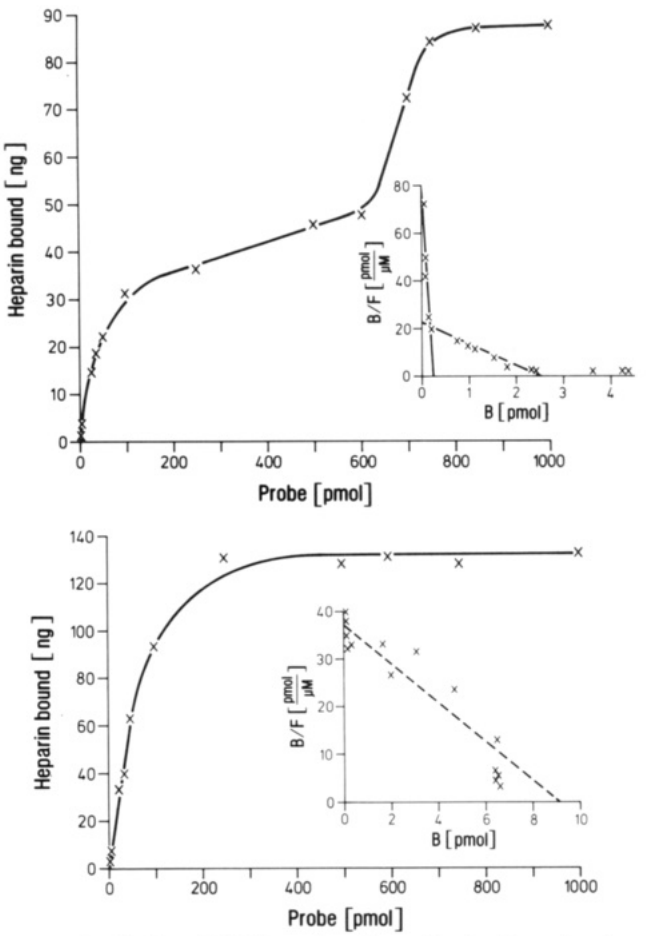

FIGURE 8: Binding of [^{35}S]heparin to 1 µg of lectin (A, top) or 1 µg of histone type VII-S (B, bottom) equivalent to H2B, in solid-phase binding assays as a function of ligand concentration. Increasing amounts of labeled heparin (1 ng–20 µg) were incubated with the immobilized lectin for 75 min to reach equilibrium and unbound heparin was washed off. Unspecific binding was found to be negligible. Insets: Scatchard transformation of the specific binding data.

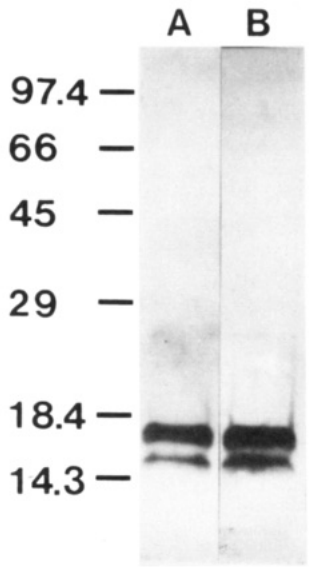

FIGURE 9: Detection of binding of heparin in the lectin fraction and in commercially available histone type VII-S, equivalent to H2B, after electrophoretic separation in the presence of SDS and electroblotting onto nitrocellulose membrane. Incubation of the blots with the probe biotinylated heparin (0.2 mg/mL) and then with avidin–peroxidase (10 µg/mL) was followed by development of the chromogenic product. Eight micrograms of protein was applied to the gel for the placenta lectin (lane A) as well as for the histone type VII-S (lane B).

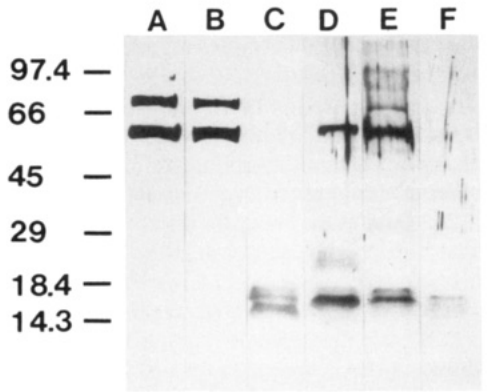

FIGURE 10: Detection of cross-reactions to an antibody, raised against the placenta lectin, in blots with specimens from placenta extract (10 µg, lane A) and after gradient elution from a heparin–Sepharose column at 0.7 M NaCl (10 µg, lane B) and at 1 M NaCl (10 µg, lane C) and with commercially available histones, namely, type VII-S (10 µg, lane D), type VI-S (10 µg, lane E), and type VIII-S (10 µg, lane F).

was found to exclusively contain cross-reactive material under standard conditions as complexes, also seen in fractions from elution of the heparin–Sepharose column at 0.7 M NaCl (see also Figure 1). The structural relationship to histones translated into immunological cross-reactivity (Figure 10). This will unmistakably have to be taken into consideration within histochemical studies. In comparison to the lectin fractions, occurrence of further cross-reactive material was also observed with the histones. An alternative approach to infer the cellular localization of the lectin is provided by subcellular fractionation, simultaneously monitored for lectin activity by hemagglutination assays.

Subcellular Localization of Lectin Activity. The hemagglutination activity with type II erythrocytes was inhibitable by heparin. Activity was found to be distributed in each of the three subcellular fractions, with no major portion being preferentially located in the nucleus (Table IV). Since the presence of DNA was totally confined to the nuclear fraction, an internal control to the quality of the fractionation was included. It is interesting to note that Western blot analysis

of material of each fraction, obtained from stepwise elution with 1 M NaCl from heparin–Sepharose, detected complex formation that is characteristic of lectin, as illustrated in Figure 10. Only the nuclear fraction after elution with 6 M guanidinium hydrochloride revealed bands with molecular weights in the range 14000–17000 (not shown).

Discussion

In this study we have further characterized the ligand-binding and structural properties of the heparin-binding lectin from human placenta. The marked resistance to disruption of heparin binding by high concentrations of NaCl and the substantial inactivation by chemical modification of arginine and lysine as well as of hydrophobic amino acids indicate involvement of further attractive forces besides ionic interactions. On the basis of similar results, establishment of a resonating hydrogen-bonding system between sulfates of the polysaccharide and arginine guanido moieties has been suggested for the interaction of the acrosomal sperm adhesion protein binding to a sulfated polysaccharide, fucoidan (DeAngelis & Glabe, 1988). This finding can by no means be generalized with respect to other heparin-binding proteins. Binding of human extracellular superoxide dismutase C to heparin was found to be at least predominantly ionic (Karlsson et al., 1988). Instead of lysine and arginine residues, heparin binding to kininogen and histidine-rich glycoprotein was strongly dependent on histidine residues (Burch et al., 1987; Björk et al., 1989). Histidine modification by diethyl pyrocarbonate, however, was not influenced by the presence of the ligand in the case of the lectin. Lack of protection by the ligand strongly argues against a direct role of the respective amino acid group in heparin binding. Since heparin binding, too, is independent of the presence of metal ions, involvement of histidine residues by complexation beyond their undisputable importance for the active protein conformation is ruled out. Therefore, our results substantiate that different mechanisms can lead to specific association of a certain polyanion to distinct classes of proteins.

Due to the various biological roles of heparin, it is not surprising that a number of specific binding proteins have been detected in membrane fractions of various cells. Already isolated proteins from a few sources show no apparent relationship to the lectin, as noted for heparin-binding proteins from smooth muscle cells, monocytes, or rat brain (Millis et al., 1985; Lankes et al., 1988; Rauvala et al., 1988; Leung et al., 1989). Similarly, a DNA-binding protein from human leukocytes displayed no relationship to the lectin (Bennett et al., 1985). On the other hand, the relationship to the heparin-binding polypeptides from rat myoid cells and human brain cannot yet be excluded (Kamo et al., 1986; Eloumami et al., 1990). Transblot experiments with labeled heparin as probe revealed a pattern of protein bands from mammalian granulosa and melanoma cells with similarities to the lectin (Biswas, 1988; Winer & Ax, 1989). These similarities extend to the properties of heparin binding, for which two classes of binding sites had been inferred for the lectin in solid-phase assays. Determination of two classes of binding sites for heparin on various tumor cells and on activated platelets supports the notion that lectin can be responsible for this cell binding (Horne & Chao, 1989; Halper, 1990).

Our observation that certain histones can also be picked up in transblot experiments with commercially available fractions advises cautious interpretation of such results. If histones are expressed on the cell surface, this can help to explain the likewise measured occurrence of a single class of heparin receptors on different cell types (Halper, 1990), as underscored by the analysis of the binding data of the solid-phase assays with histones. Indeed, it is intriguing to note the evidence that histones can be found on the surface of cells (Rekvig & Hannestadt, 1980; Jacob et al., 1984; Holers & Kotzin, 1985) and that they have other roles beyond that as a core protein of nucleosomes (Reichart et al., 1985; Aten & Behrman, 1989). In any case, the demonstrated immunological relationship between the lectin and histones emphasized that the detection of the lectin in assays, designed for histone localization, has to be ruled out for valid conclusions. Likewise, it is conceivable that histone-mediated processes such as immune complex glomerulonephritis may be influenced by the lectin. Both classes of proteins are detectable in the kidney in significant amounts after iv injection (Schmiedeke et al., 1989; Kojima et al., 1990).

Besides insolubility in 0.5 N H_2SO_4, the presence of only 2% of the hemagglutination activity in the nuclear fraction of chicken liver was judged as strong argument against a relationship of lectin and histones (Roberson et al., 1981). Subcellular fractionation of human placenta yielded an increased percentage of activity for the nuclear fraction. In view of the influence of heparin on nucleosome structure, the lectin may even be able to exert a regulatory influence in the nucleus (Brotherton et al., 1989). Herein, it may interact with nuclear proteoglycans, whose functions are not yet defined (Fedarka & Conrad, 1986). Further studies on the endogenous ligands of the lectin and its localization are required to shed light on its physiological role.

Acknowledgments

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Registry No. Lys, 56-87-1; Arg, 74-79-3; Tyr, 60-18-4; Trp, 73-22-3; His, 71-00-1; heparin, 9005-49-6.

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Regulation of the Erythrocyte Ca^{2+}-ATPase by Mutant Calmodulins with Positively Charged Amino Acid Substitutions†

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ABSTRACT: Four mutant calmodulins with site-specific charge alterations have been used to activate the human erythrocyte Ca^{2+}-ATPase. These charge alterations were accomplished either by insertion of new Lys residues or by substitution of Lys residues for Glu in two of the seven calmodulin α-helices. Two enzyme preparations, purified monomeric Ca^{2+}-ATPase and erythrocyte ghost membranes, were used with comparable results. At 100 nM Ca^{2+}, the Ca^{2+}-ATPase activity was lowered significantly by charge reversal from negative to positive in both the central α-helix and the carboxy-terminal domain. While all mutant calmodulins with charge reversal ultimately stimulated the Ca^{2+}-ATPase activity to the same extent, the concentration of mutant calmodulin required for half-maximal activation was from 36-fold (central α-helix) to 126-fold higher (α-helix in the carboxy-terminal domain) than that of the control calmodulin. There was also a significant difference in the stimulation of Ca^{2+}-ATPase activity by the different mutant calmodulins as a function of Ca^{2+} concentration, being most pronounced at submicromolar Ca^{2+} concentrations where enzyme activation by calmodulin appears to be a physiologically relevant mechanism. In contrast to the mutant calmodulins with charge reversal, mutant calmodulins in which two positive charges were added in the central α-helix activated the Ca^{2+}-ATPase in a way undistinguishable from the control calmodulin. Our results establish the relative importance of specific charged amino acids in calmodulin for the efficient activation of the enzyme and show that the interaction of the erythrocyte Ca^{2+}-ATPase with calmodulin is attenuated more by charge reversal in the α-helix of the carboxy-terminal half of calmodulin than in the central α-helix. Troponin C (TnC), another Ca^{2+}-binding regulatory protein, homologous with CaM, did not stimulate the Ca^{2+}-ATPase activity. Two experimental procedures that diminish differences in the primary structure between TnC and CaM, insertion of three amino acids into CaM or deletion of eight amino acids from TnC, did not change the function of the native molecules with respect to their ability to activate the erythrocyte Ca^{2+}-ATPase.

The human erythrocyte Ca^{2+}-ATPase is a good experimental model for plasma membrane Ca^{2+}-ATPase since in the erythrocyte there is no interference from other Ca^{2+} transporting systems, like Na^+–Ca^{2+} exchanger and gated Ca^{2+} channels or the intracellular endoplasmic/sarcoplasmic reticulum. However, regulation of this enzyme's function appears very complex. Several activation modes have been proposed, such as binding of calmodulin (CaM)[1] or acidic phospholipids, proteolytic digestion, oligomerization, and phosphorylation by kinases [for a review, see Schatzmann (1982), Eneyedi et al. (1987), Wang et al. (1989), Kosk-Kosicka and Bzdega (1988), Neyes et al. (1985), and Smallwood et al. (1988)]. We have been studying two modes of regulation, by oligomerization of Ca^{2+}-ATPase monomers and by calmodulin binding to enzyme monomers (Kosk-Kosicka & Inesi, 1985; Kosk-Kosicka et al., 1986, 1989, 1990a,b; Kosk-Kosicka & Bzdega, 1988, 1990a). Calmodulin is essential for activation of Ca^{2+}-ATPase only when the enzyme and Ca^{2+} concentrations are low: pCa below 6.25 and enzyme concentration below 15 nM (Kosk-Kosicka & Inesi, 1985; Kosk-Kosicka et al., 1989). The process of enzyme activation by CaM is complex, and believed to involve multiple substeps, including Ca^{2+} binding to calmodulin, Ca^{2+} binding to the Ca^{2+}-ATPase, binding of calmodulin to the enzyme, and finally the overall effect, enzyme activation.

In the present study, we have employed systematically perturbed, mutant calmodulins, engineered in Martin Watterson's laboratory, in which negative cluster charges were

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[1] Abbreviations: CaM, calmodulin; TnC, troponin C; MLCK, myosin light chain kinase; CaMPKII, type II calmodulin-dependent protein kinase; $C_{12}E_8$, *n*-dodecyl octaethylene glycol monoether; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane. CaM-1 has been registered in EMBL as SYNCAM. The amino acid sequences of calmodulins used in this study can be found in the EMBL Data Bank as an annotation to the accession number M11334. They were generated from the gene for SYNCAM with the following amino acid alterations: (1) CaM-8, EEE82 → KKK; (2) CaM-12A, DEE118 → 120-KKK; (3) CaM-40, E84 → K, E120 → K; (4) CaM-15, KGK insert between D80 and S81.

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