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Isolation and Characterization of a *Streptococcus pyogenes* Protein That Binds to Basal Laminae of Human Cardiac Muscle

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A 9-kDa glycosaminoglycan-binding protein (GAG-BP) was isolated from *Streptococcus pyogenes* and purified to homogeneity by affinity chromatography on heparin-agarose. The protein selectively bound to the basal laminae of human cardiac muscle and had an apparent dissociation constant of 2.5×10^{-7} M. Chemical analyses indicated that the GAG-BP was rich in alanine, lysine, and arginine (pI 9.5) and devoid of tyrosine, methionine, histidine, and half-cystine. There were no detectable carbohydrate or phosphate substituents. The amino acid sequence of the N terminus of GAG-BP showed homology with those of histone-like DNA-binding proteins of several other bacteria. Circular dichroism spectroscopy indicated that the protein was made up of 50% β-sheet and 50% β-turn and random coil in aqueous solution; however, when the protein complexed with heparin, it adopted a more ordered structure containing 25% α-helix, 50% β-sheet, and 25% β-turn and random coil. The GAG-BP cross-reacted serologically with a component of similar size in extracts of other group A streptococci and was present in the culture medium during late logarithmic growth.

The mechanisms by which *Streptococcus pyogenes* causes acute rheumatic fever and acute poststreptococcal glomerulonephritis remain inexplicable after several decades of intensive study. The pathology of both syndromes is consistent with the hypothesis that streptococcal components are released by bacteria growing at a localized site of infection (pharyngitis or pyoderma) and are carried by the blood to target organs where they accumulate by direct binding events (9, 24, 29, 35). These planted bacterial antigens elicit formation of in situ immune complexes which lead in turn to pathology through acute inflammation. As a consequence of the initial injury, tissue components (e.g., type IV collagen, laminin, heparan proteoglycans, and myosin) may become unmasked and stimulate the formation of specific autologous antibodies that exacerbate the lesions (7, 8, 14, 23).

To date, very few streptococcal components have been identified with the necessary properties to make them likely virulence factors in these diseases. Lipoteichoic acid has been found to induce structural changes in mouse renal basement membranes in vivo (21, 33). Endostreptosin, a 45-kDa cytoplasmic protein, and streptokinase, a 46-kDa extracellular nephritis strain-associated protein have been detected in immune complexes found in glomerular lesions of patients with acute poststreptococcal glomerulonephritis (6, 13, 20, 28). In addition, Vogt et al. (35) detected several unidentified cationic extracellular antigens of *S. pyogenes* in many renal biopsy specimens and suggested that ionic interactions are responsible for the attachment of these antigens to glomerular basement membranes. The pathogenic roles of these streptococcal proteins are still speculative because they have not been shown to bind directly to renal basement membranes or to cause nephritis in experimental animals.

Bergey and Stinson (1) identified two streptococcal proteins (9 and 15 kDa) that bound in vitro to basement membranes of cardiac muscle and renal tissues. This binding activity was competitively inhibited by heparin and to a lesser extent by other glycosaminoglycans. The 9-kDa protein was deposited in glomeruli of rabbit kidneys perfused in vivo with extracts of streptococci (10). In this report, we describe the purification and properties of the 9-kDa heparin-binding protein of *S. pyogenes*.

MATERIALS AND METHODS

Bacteria. *S. pyogenes* included serotypes M3 (ATCC 10389), M6 (ATCC 12348), M12 (ATCC 11434 and 12351), M24 (ATCC 10782), and M18 strain B512, M49 strain F301, and M57 strain 995 (J. Zabriskie, Rockefeller University, New York, N.Y.), and an M6 protein-deficient mutant, JRS75 (J. Scott, Emory University, Atlanta, Ga.). *S. pyogenes* M6 was selected as the primary source of surface proteins in this study. Bacteria were grown at 37°C in 60-liter batches of chemically defined broth medium (34) supplemented with 0.2% low-molecular-weight components (<5,000-kDa ultrafiltrate) from yeast extract (Difco Laboratories, Detroit, Mich.) in a pilot plant fermentor (New Brunswick Scientific). The bacteria were harvested by laminar flow filtration (Millipore Corp.) and washed with 10 volumes of phosphate-buffered saline (PBS) (0.01 M NaH_2PO_4-K_2HPO_4 at pH 7.2 containing 0.15 M NaCl).

Extraction. Bacteria were suspended in 10 volumes of 0.15 M NaCl and adjusted to pH 11.5 with 5 M NaOH. The suspension was stirred vigorously overnight at 4°C. After centrifugation, the supernatant fluid was adjusted to pH 7.2 with 5 M HCl and dialyzed against water (four times with 20 liters) for 48 h. To prevent proteolysis, we added 0.01% phenylmethylsulfonyl fluoride and 0.01% tosylphenylchloral ketone to the extract before dialysis. The extract was lyophilized and stored in a desiccator at room temperature.

Purification. The bacterial extracts were dissolved in 0.5

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M NaCl–0.01 M sodium phosphate buffer (pH 7.2) and loaded onto a column (1 by 10 cm) of heparin-agarose (Sigma Chemical Co., St. Louis, Mo.). The column was eluted at 20 ml/h with the same buffer solution until the A_{220} nm was <0.005. A 300-ml gradient of 0.5 to 1.2 M NaCl in phosphate buffer (pH 7.2) was passed through the column to elute bound bacterial components. Fractions (4 ml) were scanned at 220 nm, and the resulting peaks were pooled as indicated, dialyzed against water, and lyophilized. Streptococcal components were also recovered from culture medium. The used medium was passed through a 0.45-μm-pore-size filter (Millipore Corp.), dialyzed against water at 40°C, and lyophilized.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10 and 15% gels by the method of Laemmli (17) with a mini-gel apparatus (Bio-Rad). The broad-range molecular-weight protein kit from Bio-Rad (Richmond, Calif.) was used for standards. Proteins were stained with silver (25). Isoelectric focusing was performed in polyacrylamide gels with a flatbed apparatus (LKB, Bromma, Sweden) and ampholine gradients of pH 8 to 10.5 or 3.5 to 10 as described by LKB Products (application note 307). Western blot (immunoblot) assays were performed with rabbit antisera to selected streptococcal antigens and alkaline phosphatase-conjugated goat antibodies to rabbit immunoglobulin G (2).

Analytical methods. Protein concentrations and amino acid composition were determined after hydrolysis of samples in 6 N HCl for 24 h at 110°C. Samples were analyzed with an amino acid analyzer (model 6300; Beckman Instruments, Fullerton, Calif.). Protein was also quantified by the bicinchoninic acid assay procedure (Pierce Chemical) with bovine serum albumin (BSA) as the standard. Automated protein sequencing was performed on the purified protein with an Applied Biosystems model 471A protein sequencer (26).

Tissue preparation. Human cardiac muscle was obtained at autopsy, which was performed within 6 h postmortem. The tissue was homogenized and heat inactivated as previously described (3). Tissue fragments were collected by centrifugation and stored at −20°C. Hearts were also obtained from normal Swiss mice immediately after death, defatted mechanically, and snap frozen in liquid N_2. The frozen tissue was cut into 2-μm-thick sections with a Cryomicrotome (Reichert Instruments).

Antisera. New Zealand White rabbits were immunized intravenously with disrupted streptococci as previously described (10). Purified protein (1 mg) was injected intradermally in complete Freund's adjuvant. The animals received biweekly intravenous booster injections of 0.5 mg of purified protein. The resulting antisera were stored at −20°C. For some experiments, tissue-reactive antibodies were removed by absorption of the sera at 4°C with homogenized cardiac muscle (1 mg of tissue protein per ml of serum).

Binding assay. An enzyme immunosorbant assay (EIA) was used to measure the binding of streptococcal antigens to cardiac muscle. Homogenized human cardiac muscle was suspended in PBS and coated onto polyvinyl microtiter plates at 4°C for 18 h. Normal human serum (10%) was added to the wells and incubated for 1 h to block all exposed plastic surfaces. After being washed three times with PBS, selected streptococcal components were added to the wells and incubated at room temperature for 1 h. Streptococcal components that bound to the tissue-coated wells were detected with rabbit antiserum (1/500 dilution) to whole *S. pyogenes* or to pure binding protein. This was followed by alkaline phosphatase-conjugated goat antibodies to rabbit immunoglobulin G (1/1,000 dilution). After three washes with PBS–0.05% Triton X-100, the wells were incubated with 0.1 ml of *p*-nitrophenylphosphate (1 mg/ml) in 9.7% (vol/vol) diethylamine buffer at pH 9.8 for 15 min. The reaction was stopped with 3 N NaOH, and the A_{405} was determined with a microplate spectrophotometer (Bio-Tek Instruments, Inc., Burlington, Vt.). Wells without tissue and wells containing tissue but not treated with streptococcal components were used as reagent controls.

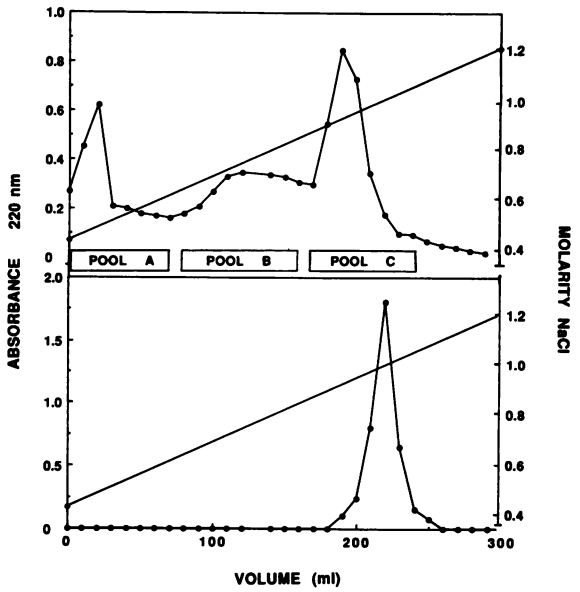

FIG. 1. Purification of streptococcal GAG-BP by chromatography on a column of heparin-agarose. Top panel, salt elution of adsorbed streptococcal components from 1 g of cell extract; bottom panel, elution of GAG-BP after loading the column with 2 mg of column pool C.

Immunofluorescence assay. Indirect immunofluorescence assays were conducted on 2- to 4-μm-thick cryocut sections of mouse and human cardiac muscle as previously described (4, 29). Briefly, tissue sections were incubated sequentially with streptococcal components, rabbit antiserum to *S. pyogenes*, and fluorescein isothiocyanate-labeled goat antibodies to rabbit immunoglobulin G. Fluorescence staining was examined with a Leitz Ortholux II microscope. For assay control, tissue sections were incubated with PBS instead of

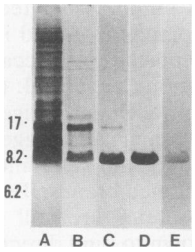

FIG. 2. SDS-PAGE (15%) and Western blot analyses of *S. pyogenes* proteins. Lanes: A, 25 μg of cell extract; B, 5 μg of column pool B; C, 5 μg of column pool C; D, 5 μg of rechromatographed protein; E, Western blot of 5 μg of rechromatographed protein with rabbit antiserum to *S. pyogenes* M6. Numbers on left show size in kilodaltons.

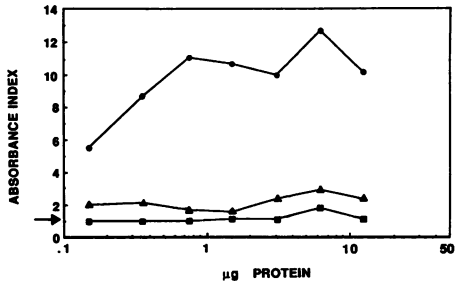

FIG. 3. Binding of streptococcal components to human cardiac muscle in EIA. Tissue-coated wells were incubated with alkali-extracted components of *S. pyogenes* (▲), *S. pyogenes* components recovered from the culture medium (■), or purified GAG-BP (●). The binding of streptococcal GAG-BP to control wells devoid of tissue is indicated by the arrow. The reactivity of muscle tissue with the primary and secondary antibodies was used as the negative control. Data are expressed as absorbance indexes: experimental value/control value.

streptococcal protein and then treated with the same serological reagents as described above.

CD. Circular dichroism (CD) spectra were measured between 190 and 250 nm with a JASCO model 600-A spectrophotometer at 25°C with 0.1-cm-path-length cells. The instrument was calibrated with 0.06% ammonium (+)-10-camphosulfonate in water, and molecular ellipticity at 290.5 nm = +7910°. Samples of glycosaminoglycan-binding protein (GAG-BP) and heparin were dissolved separately in water at 0.2 and 1.1 mg/ml, respectively. All spectra were an average of four scans and corrected for baseline drift. The conformational change induced by heparin on GAG-BP was studied with an equal mixture at these concentrations. The resulting spectrum was corrected by subtracting the spectrum of free heparin. Data were collected by an IBM PS2/30 model computer with a 1-s time constant at a scanning rate of 10 nm/s with a 0.2-nm interval. The datum points were smoothed with an 11-point smoothing function. The results were expressed as mean residue ellipticities. The estimation of secondary structure was done by the method of Provencher (27) with the program CONTIN on a Micro Vax computer.

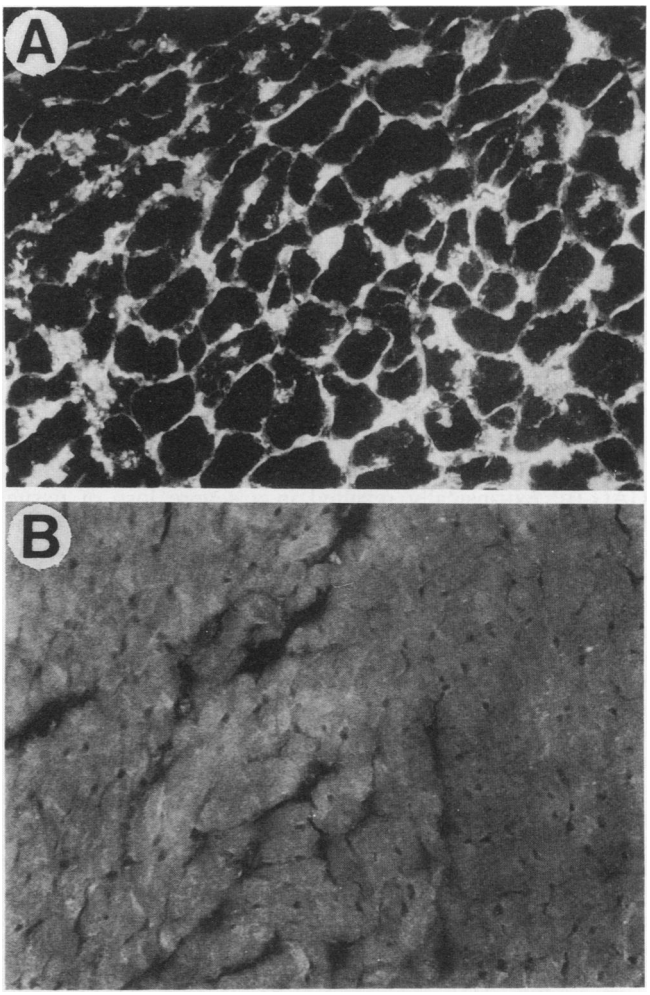

FIG. 4. Binding of GAG-BP to thin sections of murine cardiac muscle detected by indirect immunofluorescence assay. (A) Tissue cross-section incubated sequentially with 1.5 μg of purified GAG-BP, rabbit antiserum to GAG-BP, and fluorescein isothiocyanate-goat antiserum to rabbit immunoglobulin G. (B) tissue cross-section incubated with only the serological reagents (negative control). Magnification, ×400.

RESULTS

Purification. Alkali-extracted components of *S. pyogenes* were fractionated by chromatography on a column of heparin-agarose (Fig. 1). Pools B and C contained several low-molecular-weight components in SDS-PAGE analysis (Fig. 2) and exhibited binding activities for human heart tissue in EIA (data not shown). A second chromatography of these pools on the heparin-agarose column resolved the heart binding activity to a 9-kDa component that eluted at 0.9 M NaCl. Other proteins in the load sample, including the 15-kDa protein, did not bind to the column and were eluted by the loading buffer. The 9-kDa protein preparation was determined to be homogeneous by SDS-PAGE, Western blot assay with antiserum to disrupted *S. pyogenes* cells (Fig. 2, lanes D and E), and isoelectric focusing (pI 9.5). This protein was designated a GAG-BP. Typically, 4.8 mg of GAG-BP was purified from 0.8 g of crude protein that was extracted from 100 g of moist cell pellet.

Binding activity. The binding properties of the streptococcal GAG-BP were determined on human cardiac muscle in an EIA (Fig. 3). Binding of the purified GAG-BP was dose dependent and showed saturation of the tissue sites at 700 ng of protein. At similar concentrations, the unfractionated extract showed minimal binding activity. Control assays also showed that the antiserum to *S. pyogenes* cells did not react directly with human heart components under the conditions of the assay.

When incubated with thin sections of human, rabbit, or murine cardiac muscle, GAG-BP produced a reticular indirect immunofluoresence staining pattern corresponding to the connective tissue matrix of the endomysium surrounding the cardiac muscle cells (Fig. 4). GAG-BP also bound to the walls of capillaries and arterioles in the cardiac muscle (data not shown). The control tissue that was treated with BSA and antiserum to *S. pyogenes* showed no staining of these structures, indicating that heart-reactive antibodies, often present in normal rabbit sera (31), were not responsible for the immunofluorescence staining in GAG-BP-treated tissue.

Binding kinetics. All attempts to radiolabel the purified

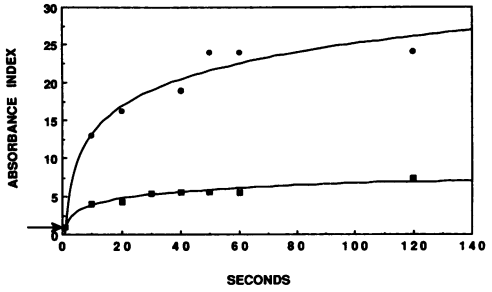

FIG. 5. Effect of time or binding of *S. pyogenes* GAG-BP to human cardiac muscle in EIA. Tissue-coated wells were incubated with 0.8 (■) or 2 (●) μg of purified GAG-BP. The arrow indicates the negative control value obtained with untreated tissue and the serological reagents.

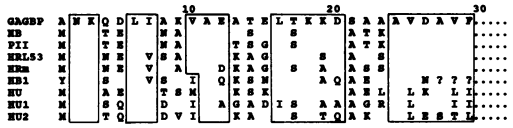

FIG. 6. Amino acid sequence of the N-terminal portion of GAG-BP and its comparison with other bacterial proteins. Only the sequence for GAG-BP of *S. pyogenes* is listed in its entirety; letters are shown for the other proteins only when they differ from GAG-BP. Several regions are boxed to emphasize high levels of homology. The proteins listed are HB of *B. globigii* (12), protein II (PII) of *Bacillus stearothermophilis* (16), HRL53 of *Rhizobium leguminosarum* (15), HRm of *Rhizobium meliloti* (18), HB1 of *Bifidobacterium longum*, HU of *Clostridium pasteurianum* (32), and HU1 and HU2 of *E. coli* (19).

GAG-BP extrinsically resulted in a severe loss of tissue binding activity which precluded using labeled GAG-BP for quantitative binding studies. Alternatively, the EIA was used to define the parameters of GAG-BP binding to human cardiac muscle. Binding was time dependent, reaching maximum levels in approximately 60 s (Fig. 5). Kinetic binding data derived from several GAG-BP concentrations were used to calculate a dissociation constant (K_d) of 2.5×10^{-7} M GAG-BP. It was also determined from Fig. 3 that 50% of the membrane receptor sites were occupied at a concentration of 2.2×10^{-7} M GAG-BP.

Composition. The amino acid composition of the GAG-BP of *S. pyogenes* is shown in Table 1. It is rich in alanine, arginine, and lysine and is devoid of tyrosine, cysteine, histidine, and methionine. Neutral and amino sugars and phosphate were not detected, indicating that the GAG-BP is unsubstituted and that there was no contamination of the sample by bacterial nucleic acids, lipoteichoic acid, or polysaccharides.

The amino acid sequence of the N terminus of GAG-BP is shown in Fig. 6 and has significant homologies with several DNA-binding proteins from other bacterial genera. For example, there is 64% homology over a 28-residue overlap with protein HB of *Bacillus globigii* and 39% over 22 residues with protein HU 2 of *Escherichia coli*. The 29-amino-acid sequence of GAG-BP is identical with that of an 8-kDa protein isolated from *Streptococcus mutans* (5).

CD. The CD spectrum of GAG-BP is characterized by a broad negative minimum at 220 nm and a sharp one around 205 nm (Fig. 7). The secondary structure estimated from the CD spectrum of native GAG-BP by the method of Provencher (27) showed 50% β-sheet and 50% other structures with negligible α-helix. In the presence of heparin, however, there was a definite change in the CD spectrum. The negative bands at 220 nm and 205 nm increased in magnitude, and a distinct positive band appeared at about 195 nm. The secondary structure estimated for this spectrum is 25% α-helix, 50% β-sheet, and 25% other structures. Thus, the presence of heparin brings about a structural alteration in the conformation of GAG-BP.

Strain distribution and localization of GAG-BP. GAG-BP was found in alkali extracts prepared from several strains and M protein serotypes of *S. pyogenes* with rabbit antiserum to GAG-BP and Western blot assays (Fig. 8). The antiserum reacted with a single band between 8.5 and 10 kDa in each antigen preparation; there was no evidence of immunological cross-reactivity between GAG-BP and other proteins in these bacteria. Some of these bacteria release the protein into the environment during growth in vitro (Fig. 8, lanes H and I).

TABLE 1. Amino acid composition of *S. pyogenes* GAG-BP

Amino acid	Mol %
Aspararic acid/asparagine	7.80
Threonine	4.60
Serine	3.20
Glutamic acid/glutamine	13.60
Proline	2.20
Glycine	7.90
Alanine	20.70
Half-cystine	0.00
Valine	6.90
Methionine	0.00
Isoleucine	5.20
Leucine	5.70
Tyrosine	0.00
Phenylalanine	5.90
Histidine	0.10
Lysine	11.90
Arginine	4.30
Tryptophan	ND[a]

[a] ND, not determined.

DISCUSSION

Our results show that the GAG-BP of *S. pyogenes* is a 9-kDa cationic protein that has binding activity for connec-

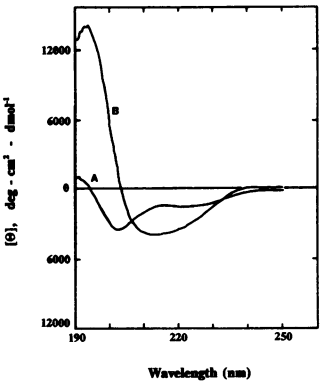

FIG. 7. CD spectra of GAG-BP (A) and a mixture of GAG-BP and heparin (B) measured in water. The spectrum of heparin (not shown) has been subtracted from the latter.

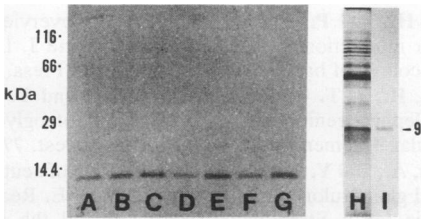

FIG. 8. Production of GAG-BP by selected strains and serotypes of *S. pyogenes*. Alkali-extracted bacterial proteins (20 μg) were analyzed by Western blot assay with 10% gels. Lanes: A, M3; B, M12; C, M18; D, M24; E, M6 (JRS75); F, M6 (D471); G, M6 (12348). Lane H is a silver stain (15% gel) of streptococcal protein (76 μg) isolated from the culture medium during the late logarithmic phase of growth of *S. pyogenes* M6. Lane I is a Western blot of 130 μg of the components described in lane H.

tive tissue proteoglycans present in basal laminae of cardiac muscle, kidney, and other tissues. We have shown previously that the binding activity is competitively inhibited by GAGs but not by the basal laminae proteins: laminin, type IV collagen, or fibronectin (1). The GAG-BP was purified to homogeneity by repeated affinity chromatography on a column of heparin-agarose. Binding appears to be mediated by multiple ionic bonds between sulfate and carboxyl groups on heparin and lysine and arginine residues on the GAG-BP. The intensity of this binding is revealed by the high-ionic-strength conditions (1 M NaCl) required to dissociate the complex. CD analysis indicated that complexing with heparin induces the GAG-BP to adopt a more helical structure such that the altered region may interact better with heparin. Since heparin is a polyanion, we assume that the region of the sequence which is more positively charged may be the segment that undergoes this conformational change. GAG-BP is rich in arginine and lysine and may have 12 positively charged residues. Of these, four lysine residues occur at the N-terminal 20 residues. However, the lysine residues in this segment are spaced so that they would not occur on the same face of the helix if this region was to adopt this structure. Thus, the N-terminal segment may not be the region of the molecule that undergoes a conformational change upon binding to heparin. Alternatively, the HU-type DNA-binding proteins, listed in Fig. 6, have a C-terminal segment in which lysine or arginine residues are spaced at every third or fourth residue, positioning which will orient the basic amino acids on one side of an α-helix to allow optimal interaction with heparin or DNA. This 20- to 23-residue segment, containing 6 to 7 positively charged residues, would amount to a 20 to 25% helix based on a protein containing approximately 90 amino acids, an amount of helix similar to that induced in GAG-BP by the presence of heparin. In addition to the basic amino acids, the dipole moment created by the formation of an α-helix enhances binding of anionic ligands at the N terminus of these secondary structures (11). The amount of α-helicity of GAG-BP is similar to that of HB protein (20%) of *B. globigii* (12) but much less than that of HU 1 (40%) of *E. coli* (18).

GAG-BP also has affinity for human cardiac muscle. The dissociation constant is similar to values reported for other bacterial proteins; for example, the exotoxin of *Corynebacterium diphtheriae* has a K_d between 1×10^{-7} and 5×10^{-8} M against a range of host cells (22). Therefore, the binding affinity of GAG-BP would be sufficient for it to selectively accumulate in organ tissues in vivo following its release by streptococci during periods of infection.

Chemical analyses indicated that the GAG-BP is an unsubstituted non-cross-linked protein with a relatively high content of cationic amino acids, giving the protein a pI of 9.5. The absence of tyrosine residues causes the protein to absorb poorly at 280 nm. The sequence of the first 29 amino acids of the N terminus of GAG-BP revealed no particular secondary structure. The sequence, representing approximately one third of the total protein, has homology with a family of DNA-binding (HU-like) proteins reported in several other genera of bacteria (12, 15, 16, 18, 19). The significance of this relationship to the possible role of GAG-BP in poststreptococcal sequalae is not clear at this time because the GAG-BP did not bind to nuclei of myocytes in the tissue sections in immunofluorescence assays nor did purified streptococcal DNA inhibit GAG-BP binding to cardiac muscle in vitro. Nevertheless, the GAG-BP was found to have DNA binding activity in vitro. The purified protein bound to single-stranded DNA in a saturable, noncooperative manner (30).

The GAG-BP is released into the milieu during the growth of *S. pyogenes*. Affinity chromatography on heparin-agarose indicated that solubilized GAG-BP may be naturally complexed with other bacterial components; the proteins in chromatography pools B and C did not bind to heparin-agarose after the GAG-BP had been removed. This suggests that the GAG-BP can act as a carrier for other streptococcal virulence factors whereby naturally occuring complexes may bind to tissue sites and exert pathologic effects synergistically. Planted bacterial antigens can also elicit formation of immune complexes in situ that trigger inflammatory processes and tissue pathology.

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