Interactions between M proteins of Streptococcus pyogenes and glycosaminoglycans promote bacterial adhesion to host cells.

Frick, Inga-Maria; Schmidtchen, Artur; Sjöbring, Ulf

Published in:
European Journal of Biochemistry

DOI:
10.1046/j.1432-1033.2003.03600.x

2003

Link to publication

Citation for published version (APA):

Total number of authors:
3

General rights
Unless other specific re-use rights are stated the following general rights apply:
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.
• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: https://creativecommons.org/licenses/

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.
Interactions between M proteins of *Streptococcus pyogenes* and glycosaminoglycans promote bacterial adhesion to host cells

Inga-Maria Frick¹, Artur Schmidtchen² and Ulf Sjöbring³

¹Department of Cell and Molecular Biology, Section for Molecular Pathogenesis, ²Department of Medical Microbiology, Dermatology and Infection, Section for Dermatology and ³Institute of Laboratory Medicine, Section for Microbiology, Immunology and Glycobiology, Lund University, Sweden

Several microbial pathogens have been reported to interact with glycosaminoglycans (GAGs) on cell surfaces and in the extracellular matrix. Here we demonstrate that M protein, a major surface-expressed virulence factor of the human bacterial pathogen, *Streptococcus pyogenes*, mediates binding to various forms of GAGs. Hence, *S. pyogenes* strains expressing a large number of different types of M proteins bound to dermatan sulfate (DS), highly sulfated fractions of heparan sulfate (HS) and heparin, whereas strains deficient in M protein surface expression failed to interact with these GAGs. Soluble M protein bound DS directly and could also inhibit the interaction between DS and *S. pyogenes*. Experiments with M protein fragments and with streptococci expressing deletion constructs of M protein, showed that determinants located in the NH₂-terminal part as well as in the C-repeat region of the streptococcal proteins are required for full binding to GAGs. Treatment with ABC-chondroitinase and HS lyase that specifically remove DS and HS chains from cell surfaces, resulted in significantly reduced adhesion of *S. pyogenes* bacteria to human epithelial cells and skin fibroblasts. Together with the finding that exogenous DS and HS could inhibit streptococcal adhesion, these data suggest that GAGs function as receptors in M protein-mediated adhesion of *S. pyogenes*.

Keywords: *Streptococcus pyogenes*; glycosaminoglycan; epithelial cells; adhesion.

Glycosaminoglycans (GAGs) belong to a group of molecules that are expressed both on cell surfaces and in extracellular matrix (ECM). These ubiquitous molecules are composed of repeating disaccharide units of amino sugars and uronic acids, forming linear sulfated polysaccharide chains (Fig. 1A). Usually, GAGs are covalently linked to a protein core in the form of proteoglycans (PGs). Based on their disaccharide composition, different classes of GAGs can be defined, including chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS) and heparin [1]. The amino sugar in CS/DS is N-acetylgalactosamine, that is linked to glucuronic acid and/or iduronic acid (IdoA), the latter found only in DS, while in HS/heparin, N-acetylgalactosamine is linked to glucuronic acid or IdoA [1]. CS/DS-containing PGs are present mainly in ECM of connective tissues, such as skin and cartilage [2]. Other PGs, such as syndecans, glypicans or various isoforms of CD44, occur on cell surfaces. Syndecans and glypicans are usually substituted with HS chains, although some members of the syndecan family can also carry CS/DS chains [3,4], whereas CD44 contains only CS or CS/HS [5].

An increasing number of microbial pathogens have been shown to depend upon interactions with GAGs for adhesion to host cells and tissues [6–8]. Specific adhesins mediating binding to GAG, and in particular to HS-chains present on cell surfaces, have been identified in viruses, parasites and bacterial species as diverse as *Borrelia burgdorferi*, *Listeria monocytogenes*, *Neisseria gonorrhoeae* and *Streptococcus pyogenes* [6–8]. For *L. monocytogenes* and *N. gonorrhoeae* recognition of HS receptors at the cell surface facilitates bacterial invasion of host cells [9,10].

*S. pyogenes* is unusual in that it is able to invade the human host through mucosal membranes as well as through the skin. The resulting infections, pharyngitis and impetigo, are usually mild, but occasionally further invasion can result in life-threatening conditions [11,12]. In order to adhere to the different tissue sites, *S. pyogenes* express a number of surface proteins that mediate interactions with host molecules [12,13]. The quantitatively dominating of these proteins, the M protein, has been traditionally regarded as a major virulence factor primarily through its ability to provide *S. pyogenes* with phagocytosis resistance [14,15]. However, the M protein is also likely to be involved in promoting bacterial adhesion to host tissue [16–22].

Here we show that *S. pyogenes* interact with several types of GAGs and that the interactions are mediated through M protein, predominantly via conserved C-repeats located...
in the COOH-terminal half of the protein. The functional relevance of the interaction is emphasized by the finding that GAGs mediate *S. pyogenes* adhesion to human cells.

**Experimental procedures**

**Bacterial strains and growth conditions**

The AP collection of *S. pyogenes* strains, representing 49 different M serotypes (Table 1), was from the WHO Collaborating Centre for Reference and Research on Streptococci (Prague, Czech Republic). The AP1 isogenic mutant, BM27.6 lacks expression of protein H [23], while BMJ71 is deficient in both protein H and M1 protein [24]. In MC25, the COOH-terminal part of the *emm1* gene of AP1 has been deleted resulting in a strain lacking cell wall anchored M1 protein [25]. This strain was kindly provided by M. Collin (Lund University, Lund, Sweden). The M1 strain, 90-226 and its M1 deficient derivative, 90-226*emm1::km*, [20] were kindly gifts from P. Cleary (University of Minnesota, Minneapolis, MN, USA). The M5 strain used is the wild-type isolate Manfredo [26]. Deletion of the *emm5* gene in M5 resulting in ΔM5, and generation of ΔM5 derivatives expressing different M5 protein deletion constructs have been described previously [27,28]. Quantitation of the expression of the truncated M protein versions was performed using the ligands fibrinogen, factor H, factor H-like protein 1 and albumin as described [28]. Quantitation was also performed using a rabbit antiserum raised against the N-terminal 23 amino acid region of the M5 protein. The M6 expressing strain JRS4 and its M negative derivative [29,30] were kindly provided by M. Caparon (Washington University, St. Louis, MO, USA). Complementation of JRS145 with

<table>
<thead>
<tr>
<th>Binding of radiolabelled DS</th>
<th>Strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 5%</td>
<td>M8, AP75, AP78</td>
</tr>
<tr>
<td>5–15%</td>
<td>M22, M37, M43, M56, M58, M59, AP72, AP73, AP74, AP76, AP77, AP79</td>
</tr>
<tr>
<td>≥ 15%</td>
<td>M1, M2, M4, M5, M6, M9, M12, M13, M15, M17, M18, M19, M23, M24, M25, M26, M27, M28, M29, M30, M31, M34, M36, M38, M39, M40, M41, M46, M47, M48, M49, M51, M53, M54, M55, M57, M60, M62, M63, M66, M69, M71</td>
</tr>
</tbody>
</table>

*Measured at a bacterial concentration of 2 × 10⁹ bacteria mL⁻¹; strains denoted AP72 – AP79 are M protein-negative strains.*

---

Fig. 1. Analysis of glycosaminoglycan-binding to *S. pyogenes*. (A) A schematic model of the CS/DS structure. A hypothetical chain, that displays a periodic, complex copolymeric structure characterized by preferential codistribution of certain disaccharide units, resulting in the generation of a block structure composed of unmodified glucuronic acid-rich and 6-O-sulfated regions interrupted by modified IdoA and 4- (or 4, 6)-O-sulfated regions (see [58]). GalNac, N-acetylgalactosamine; UA, uronic acid. (B) The binding of ¹²⁵I-labelled HS6, DS or CS to AP1 bacteria was measured at a bacterial concentration of 2 × 10⁹ bacteria mL⁻¹. (C) The binding of ¹²⁵I-labelled DS to AP1 bacteria at a concentration of 1 × 10⁹ bacteria mL⁻¹ was inhibited with various amounts of unlabelled CS, DS, HS3, HS6, or heparin.
M6 was performed by cloning of the \textit{emm6} gene in the shuttle plasmid pLZ12(spec), using a protocol described previously [23], resulting in the strain JRS145/pLZM6. Bacteria were grown in Todd-Hewitt broth (Difco, Detroit, MI, USA) at 37 °C overnight. Appropriate antibiotics were added to the culture medium when required: for BM27.6, erythromycin (1 µg·mL⁻¹); for MC25 and 90-226\textit{emm1}:km, kanamycin (150 µg·mL⁻¹); for BMJ71, tetracycline (5 µg·mL⁻¹); for JRS4 and JRS145, streptomycin (100 µg·mL⁻¹) and for JRS145/pLZM6 and the various M5 deletion constructs, spectinomycin (100 µg·mL⁻¹) was used.

Proteins, GAGs, radiolabelling and binding assay

Recombinant protein H, M1 protein and the A-S and S-C3 fragments of M1 protein were prepared as described [23,31]. Protein SIC was purified from growth media of AP1 as described [32]. Polyclonal human IgG, albumin and fibrinogen were purchased from Sigma. Chondroitinase ABC (EC 4.2.2.4) was purchased from Seikagaku Corp. (Tokyo, Japan). The GAGs, chondroitin sulfate (CS), dermatan sulfate 36 (DS36), and heparan sulfate 3 (HS3) and heparan sulfate 6 (HS6) were generously provided by L.-A˚. Fransson (Lund University, Lund, Sweden). The preparation and characterization of these compounds have been described previously [33–35]. Heparin was purchased from Sigma. Radiolabelling of CS, DS36 and HS6 with ¹²⁵I was performed as earlier described [36] and proteins were labelled with ¹²⁵I using the chloramine-T method. The ¹²⁵I was from Nordion Int. Co. (Canada), and Na₂SO₄ was purchased from Amersham Pharmacia Biotech. The binding of ¹²⁵I-labelled proteins or GAGs to streptococcal cells was analysed as described earlier [37].

Cell culture, enzymatic treatment of cells and adhesion assay

A human pharyngeal carcinoma epithelial cell line (Detroit 562; ATCC CCL 138), human foreskin fibroblasts and HeLa cells were used for studying cell adhesion of \textit{S. pyogenes} strain API or the BMJ71 mutant, lacking M1 protein and protein H. Cells were cultured in minimal essential medium with Earle’s salt (MEM; ICN) supplemented with 0.1 mM glutamine (ICN), 10% fetal bovine serum (Life Technologies) and penicillin/streptomycin (100 U·mL⁻¹/100 µg·mL⁻¹, PEST; ICN) at 37 °C in an atmosphere containing 5% CO₂ with 100% relative humidity. Analysis of the adhesion of bacteria to the cells was performed as described previously [21]. Briefly, cells grown in 24-well tissue culture plates (Costar) to near confluence were washed with MEM and infected with 2 × 10⁷ bacteria in MEM supplemented with 10% fetal bovine serum for 2 h at 37 °C. Following a washing step to remove nonadherent bacteria, trypsin (2.5 mg·mL⁻¹ in NaCl/P) was used to detach the cells from the surface and Triton X-100 (0.025% in NaCl/P) was then added to the cell suspension to lyse the cells. The amount of adherent bacteria was determined by plating appropriate dilutions of the lysates on Todd-Hewitt culture plates. For digestion of cell-associated GAGs, cells grown as above were treated with ABC-chondroitinase (50 mU·mL⁻¹) and HS lyase (1.2 mU·mL⁻¹) in MEM for 1 h. Additional enzyme was added to a final concentration of 200 mU·mL⁻¹ and 4.8 mU·mL⁻¹, respectively, and incubation was continued for another 2 h. The cell layers were then washed with MEM three times and adhesion of AP1 was determined as described. For some experiments cells were also subjected to chlorate treatment by changing the medium to NaCl-free DMEM/Ham’s F-12 supplemented with 25 mM NaClO₃ and an appropriate amount of NaCl to obtain physiological ionic strength. HeLa cells, grown to confluence, were depleted with fetal bovine serum for 16 h, washed with MEM and adhesion of bacteria, in the absence of fetal bovine serum was determined (see above).

For analysis of enzymatically released GAG chains confluent cells were labelled with [³⁵S]-sulphate (50 µCi·mL⁻¹) in sulphate-deficient F12-medium for 48 h. The monolayers were washed extensively with MEM and digested with ABC chondroitinase or HS lyase, respectively. The cell layers were then treated with 4 mM guanidinium hydrogen chloride containing 0.05 M sodium acetate, pH 5.8, containing 0.1 M EDTA, 0.01 M N-ethylmaleimide, 1% Triton X-100 and 5 µg·mL⁻¹ ovalbumin. Extracts were precipitated with three volumes of 95% ethanol and 0.4% sodium acetate and were then dissolved in SDS sample buffer and analysed by gradient PAGE (3–12%) gels. For detection of [³⁵S]-PG in the cell extracts, an Alcian Blue-binding assay (Wieslab AB, Lund, Sweden) was used [38] and the amount of radioactivity was measured by liquid scintillation. Five micrograms HS carrier was added to each sample before precipitation.

Slot binding and SDS-gel electrophoresis

Proteins were applied to nitrocellulose membranes using a Milliblot-D system (Millipore). The membranes were washed with NaCl/Tris, pH 7.5, blocked with NaCl/Tris containing 3% bovine serum albumin for 1 h and incubated for 3 h at room temperature with ¹²⁵I-labelled DS in the same buffer. After washing with NaCl/Tris + 0.05% Tween-20, the membranes were subjected to exposure on a BAS-III imaging plate and scanned with a Bio-Imaging analyser BAS-2000 (Fuji Photo Films Co. Ltd, Japan). Extracts from cells labelled with [³⁵S]-sulfate were separated on 3–12% SDS/PAGE gradient gels using the buffer system described by Laemmli [38a]. Gels were dried and the radioactivity was visualized as described above.

Results

\textit{S. pyogenes} interacts with glycosaminoglycans

As the skin is the major port of entry for invasive \textit{S. pyogenes} infections, we first studied the ability of these bacteria to bind to DS, a molecule that is abundant throughout the skin. Fifty-two M protein-expressing strains, representing 49 different serotypes, as well as eight strains that naturally express little or no M protein, were analysed for their ability to bind radiolabelled DS. The majority of the strains bound this GAG, and as shown in Table 1, there was a clear correlation between M protein expression and the ability to bind ¹²⁵I-labelled DS.

To study the ability of various GAGs to interact with streptococci, we focused initially on the M1 strain (AP1), as
this serotype is predominant in serious infections and because it can invade both through the skin and the throat. As demonstrated in Fig. 1B, AP1 bound not only $^{125}\text{I}$-labelled DS, but also radiolabelled HS6, a highly sulfated fraction of HS. In contrast, no binding of radiolabelled CS was detected. These results were substantiated by inhibition experiments with unlabelled GAGs. As expected, unlabelled DS and HS6 (and heparin) efficiently blocked the interaction between $^{125}\text{I}$-labelled DS and AP1, whereas unlabelled CS did not (Fig. 1C). Moreover, the poorly sulfated HS3 preparation only weakly inhibited binding of $^{125}\text{I}$-labelled DS to AP1 (Fig. 1C). Similar results were obtained when studying the ability of the different GAGs to bind to streptococcal strains expressing the M6 and M12 protein (data not shown).

**M proteins mediate the binding of GAGs to streptococci**

To establish the role of M proteins for the GAG interaction we again first focused on the AP1 system. AP1 expresses two members of the M protein family; protein M1 and protein H. There was a clearly reduced binding of $^{125}\text{I}$-labelled DS to the isogenic mutant strain BMJ71 that expresses very low levels of both these proteins (Fig. 2) as compared to wild-type AP1. Furthermore, both M1 protein and protein H appear to be involved in the interaction as the binding of $^{125}\text{I}$-labelled DS was reduced to isogenic derivatives of the AP1 strain lacking either of these surface proteins (Fig. 2). The significance of the M1 protein further derive from experiments with another pair of isogenic streptococci: $^{125}\text{I}$-labelled DS bound to the wild-type strain 90–226 strain that expresses M1 but not protein H, while binding to the M1-negative strain 90–226emm::km was low (Fig. 2).

The critical role of M protein for the DS interaction with *S. pyogenes* was demonstrated for two additional serotypes: $^{125}\text{I}$-labelled DS bound to strains expressing the M5 and M6 proteins much more avidly than to the M-negative variants of these strains. In contrast, complementation of the M-negative strains with genes encoding the M5 and M6 proteins, respectively, restored binding of the $^{125}\text{I}$-labelled DS probe completely (Fig. 2). In fact, the complemented strains bound even more efficiently, a result that can be explained by somewhat higher expression levels of surface-bound M5 and M6 protein on these bacteria, as confirmed with binding of $^{125}\text{I}$-labelled fibrinogen (data not shown). As with AP1, the binding of $^{125}\text{I}$-labelled DS to the 90–226, M5 and M6 strains could be inhibited with unlabelled DS, heparin, HS6 and to a lower degree with HS3, but not at all with CS, and the inhibition curves were similar to those obtained for AP1 bacteria (data not shown).

To validate the findings with purified proteins, recombinant M1 protein and protein H were applied in slots to a nitrocellulose membrane and probed with $^{125}\text{I}$-labelled DS. As a control protein, SIC, secreted by some isolates of *S. pyogenes* [32], was included. Both protein H and M1 protein bound the probe, although the interaction with protein H was of a lower magnitude, while protein SIC demonstrated no affinity for $^{125}\text{I}$-labelled DS (Fig. 3A). Furthermore, M1 protein blocked binding of $^{125}\text{I}$-labelled DS to the M1-positive but protein H negative isolate 90–226 in a dose-dependent manner, while protein H was a less efficient inhibitor (Fig. 3B). Similar results were obtained in experiments with AP1 bacteria (data not shown). Taken together, these results suggest that the interaction between *S. pyogenes* and GAGs is mediated by M protein.

**Mapping of the DS binding region in proteins M1 and M5**

To define the region responsible for the interaction with DS we first focused on the M1 protein. Radiolabelled DS was used to probe recombinant polypeptides corresponding to the NH$_2$-terminal (rA-S; Fig. 3C) and the COOH-terminal (rS-C3; Fig. 3C) parts of M1 in a slot-binding assay. As evident from these experiments, both fragments bound the probe equally well (Fig. 3D). In previous studies, we have defined the binding regions in the M1 protein for fibrinogen to the NH$_2$-terminal half (A–B3), for IgG to the central S domain, and for human serum albumin to the C-repeats (C1–C3) [31]. None of these protein ligands was able to inhibit the binding of $^{125}\text{I}$-labelled DS to the M1 strain 90–226, and bacteria that had been preincubated with plasma could still bind radiolabelled DS. While these experiments did not delineate a single region in M1 responsible for the DS-binding, they clearly suggest that interactions with GAGs can occur in an environment containing the protein ligands, such as that in secretions or exudates.

In a second attempt to depict a region in M proteins responsible for the interaction we analysed the binding of $^{125}\text{I}$-labelled DS to a series of M5 protein deletion constructs expressed on the surface of the M-negative ΔM5 strain (Fig. 4). Like M1, M5 harbours NH$_2$-terminal regions...
responsible for fibrinogen-binding (B-repeats) as well as COOH-terminal repeats that account for the interactions with albumin (C-repeats). The expression levels of the constructs was quantitated by using a rabbit antiserum directed against the N-terminal 23 amino acid region as well as binding experiments with the known M5 protein ligands factor H-like protein 1, factor H, fibrinogen and albumin [28]. These experiments demonstrated that the variant encoding the entire M5 protein from a plasmid, a somewhat higher level of M protein than the wild-type strain (data not shown). Compared to the intact M5 protein, deletion of the hypervariable NH2-terminal part (M5D\textsubscript{N}), or of the NH2-terminal part of the A-repeated region (M5D\textsubscript{AN}) resulted in a limited reduction of the DS-binding (Table 2), suggesting that amino acid residues in this part of the M5 molecule may be involved in the interaction with DS. The binding was more significantly reduced when the C-repeat region was deleted (M5D\textsubscript{C}), suggesting that these repeats are important for binding of DS to M5 expressing bacteria. The loss of binding obtained with M5 lacking both the B and C regions (M5D\textsubscript{BC}) could reflect a contribution of both regions in DS-binding, but is most likely a result of an improperly expressed M5 peptide, as deletion of the B region itself (M5D\textsubscript{B}) did not effect binding (Table 2). In summary, the results show that sequences located in the NH2-terminal part of M1 and M5 and in the C-repeated region both are required for the interaction with GAGs. The observation that the C-repeats are important for the binding of GAG to M5 fits with the fact that similar repeats are found in M proteins on virtually all strains and that most, if not all, M protein-expressing \textit{S. pyogenes} strains were found to bind 125\textsuperscript{I}-labelled DS.

**Fig. 3.** Analysis of the DS interaction with protein M1. (A) Various amounts of M1 protein, protein H and protein SIC were applied to a nitrocellulose membrane. The membrane was incubated with 125\textsuperscript{I}-labelled DS (2 x 10\textsuperscript{5} c.p.m.\textsuperscript{mL}\textsuperscript{-1}) for 3 h and the radioactivity was visualized with a Bio-Imaging analyser, BAS-2000. (B) The binding of 125\textsuperscript{I}-labelled DS to \textit{S. pyogenes} 90-226 bacteria (1 x 10\textsuperscript{9} bacteria/mL) was inhibited with various amounts of unlabelled protein M1 or protein H. (C) Schematic representation of M1 protein. Functionally important regions have been denoted; fibrinogen-binding has been mapped to the A–B3 region, IgGfc-binding to the S-domain, and albumin-binding to the C-repeats [31]. Recombinantly expressed fragments rA–S and rS–C3 are indicated. (D) The M1 protein and the A–S and S–C3 fragments of M1 were applied to a nitrocellulose membrane that was then incubated with 125\textsuperscript{I}-labelled DS (2 x 10\textsuperscript{5} c.p.m.\textsuperscript{mL}\textsuperscript{-1}) for 3 h. The radioactivity was visualized with a Bio-Imaging analyser BAS-2000.

**Fig. 4.** Schematic representation of M5 protein deletion constructs. Genes encoding the corresponding M5 constructs were cloned into the shuttle plasmid, pLZ12(spec) and expressed on the surface of the strain ΔM5 as described previously [28].

as by binding experiments with the known M5 protein ligands factor H-like protein 1, factor H, fibrinogen and albumin [28]. These experiments demonstrated that the different constructs expressed the same, or in the case of the variant encoding the entire M5 protein from a plasmid, a somewhat higher level of M protein as the wild-type strain (data not shown). Compared to the intact M5 protein, deletion of the hypervariable NH2-terminal part (M5D\textsubscript{N}), or of the NH2-terminal part of the A-repeated region (M5D\textsubscript{AN}) resulted in a limited reduction of the DS-binding (Table 2), suggesting that amino acid residues in this part of the M5 molecule may be involved in the interaction with DS. The binding was more significantly reduced when the C-repeat region was deleted (M5D\textsubscript{C}), suggesting that these repeats are important for binding of DS to M5 expressing bacteria. The loss of binding obtained with M5 lacking both the B and C regions (M5D\textsubscript{BC}) could reflect a contribution of both regions in DS-binding, but is most likely a result of an improperly expressed M5 peptide, as deletion of the B region itself (M5D\textsubscript{B}) did not effect binding (Table 2). In summary, the results show that sequences located in the NH2-terminal part of M1 and M5 and in the C-repeated region both are required for the interaction with GAGs. The observation that the C-repeats are important for the binding of GAG to M5 fits with the fact that similar repeats are found in M proteins on virtually all strains and that most, if not all, M protein-expressing \textit{S. pyogenes} strains were found to bind 125\textsuperscript{I}-labelled DS.
S. pyogenes adhere to GAGs present on eukaryotic cell surfaces

As GAGs are present at cell surfaces, we hypothesized that they can act as receptors for M protein-expressing S. pyogenes. We therefore studied streptococcal adhesion to epithelial cells or fibroblasts treated with ABC-chondroitinase that selectively removes CS and DS side-chains, or digested with HS-lyase that degrades HS side-chains. As shown in Fig. 5A, B, treatment with these enzymes successfully reduced the GAG content in membrane extracts from the treated cells, and bacterial adhesion was significantly reduced both to epithelial cells and to skin fibroblasts treated with either of the enzymes (Fig. 5C, D). The role of GAGs for adhesion was further supported by the observation that streptococci showed reduced binding to cells that had been grown in the presence of chlorate, a procedure that inhibits sulfate incorporation into GAG chains [39] (Fig. 5C, D). Moreover, preincubation of AP1 bacteria with either soluble DS or HS caused dose-dependent inhibition of the adhesion of AP1 to epithelial cells and fibroblasts (Fig. 5E). As S. pyogenes adhesion has been shown to involve binding of fibronectin [20, 40–42], we analyzed streptococcal binding to cells, depleted from this ligand by serum starvation, to exclude fibronectin-dependent adhesion. HeLa cells were used for these experiments as they do not produce fibronectin. There was an interexperimental variation in attachment, but the relative outcome of each experiment was clear. AP1 bacteria bound to cells in the absence of fibronectin, although the binding was reduced compared to the binding seen when fibronectin was included (Table 3). In conclusion, the data demonstrate that M protein-expressing S. pyogenes can use GAGs for adhesion to human cells.

Discussion

A growing number of pathogens, including bacteria, viruses as well as parasites, have been shown to use cell surface GAGs for their attachment to host cells and tissues (for references see [6–8]). The predominating GAG used by these diverse pathogens appears to be HS [3]. Although, it has been known that S. pyogenes interact with sulfated polysaccharides, for instance HS and heparin [43–46], the molecular mechanism(s) mediating such interactions has not been studied in great detail. Here, we report that S. pyogenes in addition to binding HS also bind to DS, another ubiquitous GAG, and that the binding is mediated by surface-associated M proteins.

It is assumed that binding of eukaryotic proteins to various GAGs depends on electrostatic interactions between the negatively charged sulfate groups of the GAG chains and positively charged regions of the ligand. Typically, the heparin-binding domains of known GAG-binding proteins are rich in basic amino acids that are usually clustered, although well-defined consensus sequences that

---

Table 2. Localization of the DS-binding region in M5 protein.

<table>
<thead>
<tr>
<th>M5 protein constructs(^a)</th>
<th>Binding of radiolabelled DS(^b) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M5</td>
<td>50.3 ± 4.3</td>
</tr>
<tr>
<td>M5AN</td>
<td>41.4 ± 2.8</td>
</tr>
<tr>
<td>M5AA</td>
<td>48.9 ± 1.4</td>
</tr>
<tr>
<td>M5AN</td>
<td>41.6 ± 0.5</td>
</tr>
<tr>
<td>M5AC</td>
<td>62.5 ± 2.0</td>
</tr>
<tr>
<td>M5AB</td>
<td>49.5 ± 0.8</td>
</tr>
<tr>
<td>M5AC</td>
<td>30.4 ± 0.1</td>
</tr>
<tr>
<td>M5ABC</td>
<td>1.7 ± 0.3</td>
</tr>
</tbody>
</table>

\(^a\) The M5 protein deletion constructs are shown in Fig. 4; \(^b\) measured at a bacterial concentration of 2 \(\times 10^9\) bacteria/mL.
Table 3. Adhesion of *S. pyogenes* to HeLa cells. Wild-type AP1 bacteria and mutant AP1 lacking surface-bound, M1 protein and protein H (BMJ71) was analysed for adhesion to HeLa cells. Values are mean ± SD from four independent experiments with triplicate samples.

<table>
<thead>
<tr>
<th>Strains</th>
<th>+ Fibronectin</th>
<th>– Fibronectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP1</td>
<td>20.7 ± 11.3</td>
<td>10.7 ± 4.9</td>
</tr>
<tr>
<td>BMJ71</td>
<td>2.0 ± 0.6</td>
<td>2.4 ± 1.4</td>
</tr>
</tbody>
</table>

* Supplied via fetal bovine serum.

account for these interactions have not been identified [47]. While M proteins lack regions showing significant homology with other GAG-binding proteins, they do contain regions that are rich in basic amino acids both in the NH2-terminal and in the C-repeat region of M1 and M5 proteins, both of which demonstrated affinity for DS (Fig. 3 and Table 2). However, the M protein–GAG interaction seems to be dependent not only on electrostatic attractions, but also on the presence of IdoA residues in the GAG chain, as M protein failed to bind CS. CS and DS differ mainly in the epimerization of the uronic acid (glucuronic acid in CS and IdoA in DS; Fig. 1A) and IdoA is also present in significant amounts in HS6 and heparin. The presence of IdoA results in an increased flexibility of the chains, a property that has been shown to be important for GAG interactions also with other proteins [48], such as antithrombin, glycoprotein gD from herpes simplex virus, fibroblast growth factor-1 and fibroblast growth factor-2 [49]. As the IdoA in DS and HS/heparin may be 2-O-sulfated [50], it is also possible that additional modifications of the DS and HS polymers could be required for the binding to *S. pyogenes*.

It has been known for many decades that M proteins are critical for the ability of *S. pyogenes* to resist phagocytosis [51] and much effort has been invested in the analysis of the molecular mechanisms explaining this property. However, in spite of being by far the most abundant surface protein expressed on *S. pyogenes*, relatively little attention has been paid to its putative role as an adhesin. In fact, only a few examples where the direct binding of M protein to a specific cell surface structure mediating streptococcal-host cell contact have been described until now, namely the binding of M6 streptococci to keratinocytes through CD46 [18,19], and to human pharyngeal cells through sialic acid-containing receptors [22]. Apart from the direct interactions, it is likely that M proteins, along with other surface-bound proteins including protein F/protein Sfb [42,52], can promote cell adhesion indirectly through first binding a circulating ligand such as fibronectin [20,41]. However, while such interactions may be relevant for bacterial adhesion to host cells under conditions where such proteins are available, it appears likely that the bacteria must also possess mechanisms whereby adhesion can occur also in the absence of intermediate host ligands. The data presented here suggests that M protein-mediated binding to GAGs is one such mechanism.

Apart from facilitating the interaction with host cells and tissues, it is conceivable that streptococci could benefit from GAG-binding through other pathways. One such possible benefit would be to exploit the ability of certain GAG fragments to inactivate host antibacterial peptides [53,54]. Thus, *S. pyogenes* secrete a cysteine proteinase capable of releasing DS fragments with such an activity from DS-containing PGs [54]. It can therefore be speculated that a microenvironment favouring streptococcal survival could be generated by the action of the cysteine proteinase on M protein-bound GAGs. The cysteine proteinase is also known to release fragments of M protein from the bacterial surface [55]. Therefore, it is possible that M protein-bound GAGs could modulate such an activity. In this context, it is also interesting that, in response to tissue injury or inflammation, syndecan shedding with release of soluble HS proteoglycan ectodomains has been suggested to occur [56]. Moreover, soluble GAGs are abundant in wounds and DS constitutes a large proportion of these GAGs [57]. Therefore, it can also be speculated that in such environments, *S. pyogenes* bacteria could benefit through interactions with DS or HS. Furthermore, because of their multiple binding activities, it is also possible that GAGs or GAG fragments remaining bound to the streptococcal surface could mediate binding to proteins involved in host defence. Known relevant ligands for GAGs include growth factors, cytokines and other mediators of inflammation [3]. Hence, trapping of these mediators could provide the bacteria with means to modulate the local response to the pathogen.

Acknowledgements

We are indebted to I. Gustafsson and U. Johannesson for expert technical assistance. This work was supported by the Swedish Research council (grants no. 7480, 9926 and 13471), the Royal Physiographic Society in Lund, the foundations of Crafoord, Kock, Bergvall, Österlund, and HANS MEDICAL AB.

References


