REVIEW ARTICLE

Glycosaminoglycan-binding microbial proteins in tissue adhesion and invasion: key events in microbial pathogenicity

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Glycosaminoglycans such as heparin, heparan sulphate and dermatan sulphate, are distributed widely in the human body. Several glycosaminoglycans form part of the extracellular matrix and heparan sulphate is expressed on all eukaryotic surfaces. The identification of specific binding to different glycosaminoglycan molecules by bacteria (e.g., *Helicobacter pylori*, *Bordetella pertussis* and *Chlamydia trachomatis*), viruses (e.g., herpes simplex and dengue virus), and protozoa (e.g., *Plasmodium* and *Leishmania*), is therefore of great interest. Expression of glycosaminoglycan-binding proteins depends on growth and culture conditions in bacteria, and differs in various phases of parasite development. Glycosaminoglycan-binding microbial proteins may mediate adhesion of microbes to eukaryotic cells, which may be a primary mechanism in mucosal infections, and are also involved in secondary effects such as adhesion to cerebral endothelia in cerebral malaria or to synovial membranes in arthritis caused by *Borrelia burgdorferi*. It has been suggested that they may enhance intracellular survival in macrophages. Microbial binding of heparin may interfere with heparin-dependent growth factors. Whether or not glycosaminoglycan-binding proteins mediate invasion of epithelial cells is a matter of controversy. Heparin and other glycosaminoglycans may have potential uses as therapeutic agents in microbial infections and could form part of future vaccines against such infections.

Introduction

A number of bacterial, viral and parasitic pathogens have been shown to express surface proteins that interact with specific glycoconjugates on cell surfaces [1, 2]. Pioneer studies on haemagglutinating *Escherichia coli* and other enteric organisms, and haemagglutinin and sialidases of influenza virus, were published several decades ago. Development of specific glycoconjugate receptor analogues against influenza virus, as well as receptor analogues to gal-1-4 β -gal conjugate carbohydrate receptor structures of the blood group antigen for uropathogenic *E. coli*, has encouraged much later research to define specific microbial surface haemagglutinins and other carbohydrate-binding proteins, generally called lectins, as targets for anti-adhesion therapy.

Glycosaminoglycans (GAGs), i.e., heparan sulphate, dermatan sulphate, chondroitin sulphates, keratin

sulphate, heparin and other sulphated molecules, and hyaluronic acid which is unsulphated, are found ubiquitously in the human body. They form a major part of the extracellular matrix (ECM). Heparan sulphate is expressed on the surface of eukaryotic cells and heparin is present in mast cells. Cell surface GAG molecules mediate many cellular processes. Among these are cell–ECM adhesion [3, 4], cell migration and growth, and various cell-to-cell signalling processes [1, 5]. They also bind growth factors, growth factor-binding proteins, apolipoprotein, proteases and protease inhibitors [6]. Many pathogenic bacteria, viruses, fungi and parasites have learnt to use cell surface glycolipids, glycoproteins and GAGs as receptor molecules for cell attachment, as well as eukaryotic cell invasion and intercellular migration processes. These interactions usually involve specific surface proteins of micro-organisms, named adhesins. As these proteins commonly interact with specific carbohydrate structures on glycoconjugates, they can also be defined as microbial surface lectins that enable the microbes to initiate an infectious process.

Microbial binding to glycolipids and glycoproteins by specific surface lectins, often with haemagglutinating (HA) activity, has been discussed extensively elsewhere [7]. In contrast, binding to cell surface and ECM GAGs has been studied poorly, despite the fact that well-defined interactions with heparan sulphate and chondroitin-4-sulphate were described many years ago for herpes simplex virus [8] and, more recently, for bacterial pathogens such as *Helicobacter pylori* [9], *Staphylococcus aureus* [10] and *Streptococcus pyogenes* or group A streptococci [11] (Table 1). Early observations showed that *Trypanosoma cruzi* possessed a cell-surface protein that bound to specific heparan sulphate molecules on macrophages. This triggers cell uptake and adhesion to specific ECM molecules involved in phagocytic processes, such as fibronectin (Fn) [12]. Another important human parasite, *Plasmodium falciparum*, was shown to bind two specific ECM molecules, Fn and thrombospondin (TSP), as well as heparin, heparan sulphate and chondroitin sulphate A [13–17]. In 1996 and 1997, two intracellular pathogens, *Mycobacterium tuberculosis* and *Listeria monocytogenes*, were shown to bind heparan sulphate by specific surface molecules [18, 19], and the first experimental data were presented to prove that binding to this GAG molecule on macrophages and other cells may trigger cellular uptake [19].

This review focuses on GAG molecules as adhesion receptors for bacteria, viruses and parasites. As GAG-structures are terminal carbohydrate structures linked to protein cores buried in cell membranes in the ECM, as well as in intracellular granulae, specific microbial GAG-binding proteins (GAGBPs) may be involved in cell adhesion, cell uptake and invasion, and in various intercellular and ECM tissue penetration processes [1]. Interestingly, two bacterial pathogens, *H. pylori* and *Neisseria gonorrhoeae*, bind heparin and heparan sulphate [9, 20] in the same way as some pathogens commonly defined as extracellular microbes, i.e., *Borrelia* spp. and *Treponema pallidum* [21, 22]. These

pathogens may adapt to multiply in the ECM in various body tissues, without triggering uptake by macrophages and other phagocytes, or by other cells. From current knowledge of the most extensively studied GAG–microbe interactions, an initial cell-binding event must be followed by binding to other cell-surface glycoconjugates, most likely specific cell-surface glycolipids, as in the case of herpes simplex and related viruses such as cytomegalovirus (CMV) and dengue virus [23]. One recent report suggests that prions may also use heparan sulphate or other similar cell surface and ECM molecules, such as laminin, in cellular interactions in the brain and other body tissues. This information should stimulate much research on the interplay of possible multiple glycoconjugate microbial adhesion events in determining the first and later stages of various viral, bacterial and parasitic infections [24].

Glycosaminoglycan and proteoglycan chemistry

Several cell membrane proteoglycans have single transmembrane-spanning domains in a type I orientation [25], with syndecan-1 as the prototype. The members of this family of so-called core proteins are fibroglycan-syndecan and ryudocan (Table 2). Each of these molecules has conserved attachment sites for 3–5 GAG chains. The syndecans exemplify hybrid proteoglycans as they contain mixtures of the two major types of GAG chains found in animal cells, heparan sulphate and chondroitin-4 sulphate. Other cell-surface proteoglycans include hybrid molecules of β -glycan and a spliced form of the CD 44 molecule (epiglycan), chondroitin sulphate proteoglycans and thrombomodulin [1]. The other major family of proteoglycans is named glypicans, as the members possess glycosylphosphatidylinositol membrane-spanning domains such as glypican-1 and K glypican. Unlike the syndecans, the glypicans appear to contain only heparan sulphate chains. Interestingly, expression of these proteoglycans

Table 1. Glycosaminoglycan binding by microbes

Organism	Mol. wt (kDa)	GAG-binding molecule
<i>Staphylococcus aureus</i>	60	Heparin, heparan sulphate
<i>Streptococcus pyogenes</i>	...	...
<i>Bordetella pertussis</i> (FHA, fimbriae)	...	...
<i>Haemophilus influenzae</i>	...	...
<i>Chlamydia pneumoniae</i>	...	...
<i>Borrelia burgdorferi</i>	...	...
<i>Mycobacterium tuberculosis</i>	28	Heparin
<i>Listeria monocytogenes</i>	...	Heparin, heparan sulphate
<i>Neisseria gonorrhoeae</i> (Opa)	...	Heparin
<i>Helicobacter pylori</i>	57	Heparin, heparan sulphate
<i>Yersinia enterocolitica</i> (LcrG)	...	Heparin, dextran sulphate
<i>Mycoplasma pneumoniae</i>	...	Dextran sulphate
<i>Leishmania donovani</i>	...	...
<i>Trypanosoma cruzi</i> (penetrin)	...	...
<i>Plasmodium falciparum</i>	...	...
Herpes simplex virus (gB, gC)	...	Heparan sulphate
Cytomegalovirus	30, 34	Heparan sulphate
Dengue virus	...	...
Human immunodeficiency virus	...	...

Table 2. Core proteins of glycosaminoglycans

Core protein	Occurrence
Serglycin	Haematopoietic cells; complex with basic proteases
Syndecan-1	Epithelia, endothelia; transmembrane core protein
Syndecan-2 (fibroglycan)	Fibroblasts
Syndecan-3	Nervous tissues, mesenchymae
Syndecan-4: Amphiglycan	Fibroblasts
Ryudocan	Vascular endothelia
Glypicans	Eukaryotic cells
Perlecan	Basement membranes, cartilage
CD44 Epiglycan	Epidermis
β -Glycan	Receptor for TGF β
Agrin, Testican	

occurs in a tissue-specific manner, although their expression overlaps in some cell types and in the apical and basolateral membranes of polarised epithelial cell monolayers. It is noteworthy that heparin is cleaved from the serglycin core protein in mast cells. Free heparin chains form complexes with basic proteases and peptidases which are packed in secretory granules. Upon degranulation, these are released and the enzymes dissociate. In contrast, heparan sulphate is secreted intact from cells [5].

Proteoglycans exhibit an enormous structural heterogeneity caused by great variations in glycosylation patterns, variations in glycan chain lengths of GAG chains, and variation in the extent and pattern of sulphation and epimerisation [1, 25]. Various proteoglycans are purified by methods based on different charge properties. Thus, chondroitin sulphate C and A, usually with one SO₄ residue/disaccharide unit, differ from chondroitin sulphate B (syn. dermatan sulphate), which contains more sulphate and iduronic acid instead of the galacturonic acid found in chondroitin sulphate A, C and heparan sulphate. Heparan sulphate chains usually contain 0.8–1.4 sulphate groups/disaccharide unit, while heparins synthesised in intracellular granulae of mast cells contain ≥ 2.4 sulphate groups/unit [25]. In both heparin and heparan sulphate, these sulphate residues occur at the amino groups and the C-6 hydroxyl groups of glycosamine residues, at C-2 of iduronic acid and glucuronic acid, and sometimes at the C-3 OH position of glycosamine residues. Various modifications occur to a greater extent in heparin than in heparan sulphate (e.g., >80% of glycosamine residues are N-sulphated compared to <60% of heparan sulphate). The content of iduronic acid and the degree of O-sulphation also vary more in heparin.

Intramolecular variations in the GAG chain, chain length and degree of sulphation define how these molecules interact with specific ECM proteins such as Fn, collagen type I (Cn I), laminin, vitronectin [5, 26] and, most likely, other molecules such as nidogen, which interacts specifically with *Leishmania* parasites [27]. Interestingly, so-called heparin-dependent growth

factors, the acidic and basic growth factors and platelet-derived growth factor (PDGF) also bind to distinct pentasaccharide units with specifically positioned 3-O sulphated glucosamine residues, shown also to bind to antithrombin III [5]. Thrombin, on the other hand, binds to heparin through non-specific electrostatic association with disaccharides of the heparin chain. A number of serum proteins, such as apolipoprotein and lipoprotein lipase, contain specific peptide sequences of basic and non-polar amino acids that define specific heparin-binding motifs. A heparan sulphate-binding peptide derived from *P. falciparum* [28, 29], a chondroitin sulphate-binding protein [17] and the N-terminal region of an actin-binding protein, ActA, of *L. monocytogenes* [19] have been shown to interact with similar basic synthetic peptides constructed on specific sequences of human serum heparin-binding proteins.

How should specificity in microbial GAG-binding and specific GAGBPs be defined? The best studied GAG-binding system is probably the binding of heparin to antithrombin [5], and much can be learnt from these studies. Several microbial cell-surface proteins interact with highly negatively charged sialic acid-containing glycoconjugates (S fimbriae, K99, CFAI and various CFA II surface lectins). Heparin and some other GAG molecules also have a negatively charged surface *in vivo*.

Vaheri *et al.* [30] were probably the first to define a heparin-sensitive microbial cell-binding event in studies on the interaction of herpes simplex virus with a monolayer of HeLa cells in tissue cultures. However, the precise mechanism by which heparin blocks cell adhesion of microbes has been difficult to elucidate because of the lack of commercially available purified heparins and heparin-derived fragments, now in clinical use in drugs such as Fragmin® (KABI-Pharmacia, Stockholm, Sweden) [31, 32]. Such fragments of heparin and other GAG chains, as well as those from various natural sulphated carbohydrate polymers such as fucoidan and some carageenans, often called 'heparinoids', are used as inhibitors to define the specificity of sulphate interactions in the binding of viruses, bacteria and parasites to specific tissue culture cells and ECM derived from cells grown on various biosurfaces. A mixture of laminin, collagen type 4 and GAGs (Matrigel®; Collaborative Res., Bedford, MA, USA) has been useful in defining heparin-sensitive and heparin-insensitive interactions with ECM-associated proteins and proteoglycans [3]. Moreover, various specific glycosidases, such as heparinase, heparitinases and chondroitin sulphatases, now available from Glycosystems (Oxford) and a few other companies, were used as 'receptor-destroying enzymes' in an analogous manner to the use of various sialidases (neuraminidases) to destroy sialoglycoconjugate cell receptors for influenza virus, Newcastle disease virus and *T. cruzi* [33].

When cells are cultured in a medium with inhibitors of GAG synthesis (e.g., xylose residues linked to a hydrophobic glycon such as β -D xylosides), unglycosylated proteoglycans appear on the cell surface. Alternatively, cells can be grown in a medium deficient in organic sulphate supplemented with sodium chlorate as an inhibitor of GAG sulphation [1]. However, use of the β -xyloside or the chlorate method will also cause undersulphation of other sulphated cell-surface glycoconjugates such as various glycolipids or sulphatides. Mutant Chinese hamster ovary cells and mutants of mouse L cells deficient in heparan sulphate and chondroitin sulphate have also been used to define GAG binding of CMV and dengue virus [23].

Binding assays

Radiolabelled proteoglycans and GAGs can be prepared from cells grown with radioactive GAG precursors such as $^{35}\text{SO}_4$ or $6\text{-}^3\text{H}$ glycosamine [5]. Purified heparins and other GAG molecules can be iodinated to quantify GAG-binding surface molecules of intact microbes in an analogous manner to that used for determining Fn-binding surface proteins (FnBPs) and other ECM-binding proteins [3]. This method is also applicable to the discrimination of specific from non-specific binding in competition experiments that pre-incubate virus, bacterial, or parasite suspensions with non-radiolabelled heparan sulphate, Fn and other ECM-associated molecules and cell-surface glycoconjugates. Cell-surface extracts of micro-organisms binding ^{125}I -labelled GAG molecules or solubilised virus proteins can be used in similar inhibition assays. The use of glycosidases, as described above, is helpful. Such experiments should also be performed with purified GAG-binding proteins to discriminate binding to specific proteins from binding to other surface molecules that can bind sulphated and non-sulphated charged molecules such as RNA and nucleotides to 'histone-like' intracellular proteins and heat-shock or stress proteins (HSP) of the GroEl class (60, 70 and 90 kDa). These include those found in *H. pylori* [34], chlamydiae [35, 36], *Candida albicans* [37, 38] and other microbes that bind heparin, other GAGs, sulphatides and sialylated glycoconjugates. In *H. pylori*, a surface-associated 17-kDa protein has been reported to bind all these charged molecules and sulphated gastric and intestinal mucins (B. Appelmeik, personal communication; T. Wadström, unpublished observation). Moreover, as certain phosphated molecules, e.g., ATP, bind to specific binding domains of protein toxins such as *Clostridium difficile* type A toxin (S. P. Borriello, unpublished observation), a wide range of phosphated, sulphated and sialylated compounds should be investigated to define specificity in the binding properties of GAGBPs before defining GAG proteoglycan-specific cell and ECM receptors for various microbes [23]. Interestingly, a prion molecule has been reported to bind specifically to sialylated glycopeptides derived

from laminin in a similar fashion to that reported for sialic acid-specific haemagglutinins or lectins of *H. pylori* and S-fimbriate *E. coli* [24]. Studies on heparin binding by the major fimbrial subunit, Fim2, of the respiratory tract pathogen *Bordetella pertussis* used maltose-binding protein-Fim2 fusion proteins, deletions thereof, and site-directed mutagenesis to show that the basic amino acids lysine and tyrosine were important for binding to sulphated sugars [39].

Biotinylation of heparin and other GAG molecules has been used as a tool in several investigations [9]. Stearns *et al.* synthesised three heparin moieties and used them as probes to detect heparin-binding proteins (HEBPs) [40]. This may be a valuable alternative method, particularly when the ligand is sparse. Heparin and other GAG molecules are highly negatively charged. Thus, it is important to elucidate whether binding is a result of charge interactions. If binding occurs to sulphate, it is likely that binding will be mediated to several of the sulphated GAGs. Keller has emphasised the importance of defining specificity of heparin or heparan sulphate – protein interactions [41]. Finally, the pH optimum should be defined, as well as the ionic strength and affinity constant (K_d) by Scatchard plot analysis.

Analysis, with calculation of the number of GAG-binding molecules, can be carried out if the molecular mass of these molecules has been defined by purification, e.g., by affinity chromatography or by identifying GAG-binding molecules in SDS-PAGE-nitrocellulose membrane blotting. Table 3 [42] shows the results from ^{125}I -heparin binding to specific surface molecules of *S. aureus*, *Str. pyogenes* and *H. pylori*. It should be noted that the *S. aureus* and *Str. pyogenes* molecules bind with low affinity and low specificity because various charged molecules partially inhibit ^{125}I -heparan sulphate binding; this is in contrast to results with *H. pylori* [42].

Glycosaminoglycan binding in microbial adhesion to mucosal surfaces

Examination of the binding properties of ^{125}I -heparan sulphate with sialoglycoconjugates, and haemagglutina-

Table 3. Characteristics of ^{125}I -heparan sulphate-binding by three bacterial species

Feature	<i>H. pylori</i>	<i>S. aureus</i>	<i>Str. pyogenes</i>
Optimal ionic strength (M NaCl)	9×10^{-9} M	3×10^{-5} M	9.8×10^{-6} M
Optimal pH	4	6	4
K_d	0.5	0.15	0.5
Mol. wt of heparan sulphate-binding proteins (kDa)	120, 72, 35	66, 60	63, 58

Data updated from [42].

tion assays with fetuin, glycoporphins and hyaluronic acid in combination with a great number of 'heparinoids' as potential HA inhibitors, showed that heparin-inhibitable 125 I-heparan sulphate binding occurs with all strains of *H. pylori* tested, including so-called class I and II strains as based on the *cagA* pathogenicity 'island' [43]. Likewise, expression of sialic acid haemagglutinin and other sialic acid-binding surface lectins (SALs; present in *c.* 50% of strains of both class I and II; T. Wadström *et al.*, unpublished results) did not influence binding of heparin. Interestingly, *Bord. pertussis* expresses a filamentous haemagglutinin (FHA) that binds heparin and other GAG molecules by its C-terminal [44], whereas the B subunit of pertussis toxin binds specific sialoglycoconjugates [45]. However, there are studies suggesting that other surface proteins of the *Bord. pertussis* FHA recognise both sialo- and sulphated cell glycoconjugates, as such compounds inhibit haemagglutination, unlike *H. pylori* as discussed above [46]. Interestingly, other *Helicobacter* spp. isolated from animals, such as *H. felis* and *H. mustelae*, commonly express GAG-binding surface molecules, but not SALs (T. Wadström *et al.*, unpublished results). Several high mol. wt proteins of *Haemophilus influenzae* show significant homology to *Bord. pertussis* FHA and bind heparan sulphate-like molecules in the respiratory epithelium [47]. A common motif involved in this binding includes a peptide sequence rich in basic amino acids flanked by hydrophobic residues (Table 4) [19] and two distant regions of a primary sequence. These may be brought together by a secondary structure to form a binding site. Genomic studies comparing the *H. pylori* genome [48] and *Hae. influenzae* have not so far revealed FHA-like proteins involved in heparin binding (M. Utt, unpublished observation). Further analysis of the molecular properties of the first *H. pylori* HEBP (57 kDa) will reveal if this protein belongs to a new class of microbial GAGBPs, despite the fact that *H. pylori* and *Hae. influenzae* are rather closely related organisms according to genomic analysis [48]. Interestingly, HEBP of *M. tuberculosis* and *M. bovis* is also a haemagglutinin that agglutinates rabbit erythrocytes [18].

With *Mycoplasma pneumoniae*, dextran sulphate has been shown to inhibit adhesion to pulmonary epithelium [49], suggesting that binding of dextran sulphate would mediate adhesion. This has not been followed up. Klotz and Smith [37] were the first to describe the influence of various GAG molecules on the binding of *C. albicans* to different ECM proteins. In a series of experiments it was shown that GAG molecules and dextran sulphate bind primarily to heparin-binding ECM molecules such as Fn and, in so doing, cover or mask potential binding ligands in ECM for *C. albicans*. Dextran sulphate, which is more highly charged than heparin, did not show any effect, indicating that specific interactions occur between heparin or heparan sulphate and subendothelial ECM [37]. However, a GAG-binding protein has not yet been identified in *C. albicans*.

Bergey and Stinson [11] described heparin-inhibitable binding to ECM basal membranes of *Str. pyogenes* and *Str. mutans*, and proposed that these binding properties were possible virulence traits in specific infections caused by these pathogens. Heart tissue GAG binding could target the attachment of microbes to heart valves in subacute endocarditis, and binding to the basement membrane in kidneys could be involved in glomerulonephritis following *Str. pyogenes* infections. These early observations, as well as the observation that GAG-like molecules and, possibly sulphated glycolipids bind to organisms grown in brain heart infusion broth encouraged a comparison of the influence of various media and culture conditions on expression of GAG binding. GAG-binding proteins of *H. pylori* were expressed optimally in GabCamp broth, unlike production of sialic acid-specific and other *H. pylori* HAs and Lewis-binding proteins which were expressed optimally on solid media such as GabCamp agar [50, 51]. The opacity factor or Opa adhesin of *N. gonorrhoeae* was expressed when the bacteria were grown in polysaccharide-free medium [52]. Growth in serum or on agar resulted in coverage of the bacterial cells with polysaccharides binding to the Opa adhesin. For the recently described GAGBPs of *M. tuberculosis* [18] and *L. monocytogenes* [19],

Table 4. Heparin-binding sequences

Organism or molecule	Protein	Amino-acid sequence
<i>Plasmodium</i> spp.	CS protein (pepPfl)	QCNVTCGSIRVRKR
<i>Listeria monocytogenes</i>	ActA	Undefined*
Herpes simplex virus	gp C	RXXXRCFRXXXR
Herpes simplex virus	gp B	KPKKNKKPKPPKP
Human immunodeficiency virus	gp 120	RKSIRIQRGPGRAEVTIGKIG
<i>Chlamydia trachomatis</i>	MOMP	Undefined
<i>Helicobacter pylori</i>	GAGBP	Undefined
Laminin	...	RIQNLLKITNLRKIFVK
Antithrombin III	...	KLNCRLYRKANKSSKLVSANR
Endothelial cell growth factor	...	KKHEAKNWFVGLKKNKNGSCKR
Apolipoprotein E	...	RKLRKRLRLDAEDLQKRLAV

*The sequence in *L. monocytogenes* is probably very similar to that of *Plasmodium* spp., as a synthetic peptide of the heparan sulphate-binding ligand, pepPfl, inhibited attachment of *Listeria* to epithelial cells, and a similar motif was found in the N-terminal of ActA [19].

similar studies on expression of these proteins *in vitro* are lacking. Studies on expression *in vivo* are also needed. Whereas heparan sulphate and other GAG molecules have been shown to block chlamydia infection *in vitro*, chlamydia infection was not blocked in a mouse vaginal model of infection [53].

Blecker *et al.* had already shown in 1969 that heparin inhibits binding of *Chlamydia psittaci* to tissue culture cells in monolayers, and that dextran sulphate also inhibited binding [35]. More recently, components of the outer-membrane cell envelope of chlamydiae have been shown to bind heparan sulphate and other GAG molecules [54, 55], but specific biovariants of the trachoma-*Lymphogranuloma venereum* (LGV) group also express neuraminidase-sensitive eukaryotic cell receptors [56]. However, as heparin inhibits cell binding of all chlamydiae, the basic mechanism of cell attachment is likely to be common in the genus, thus including 'new' pathogens such as *Ch. pneumoniae* or TWAR [56]. In brief, cell attachment and infectivity is trypsin-sensitive and is reduced following mild heat treatment of elementary bodies, but cell invasion by these organisms probably also involves other glycoconjugate receptors [57]. Moreover, chlamydiae seem to synthesise heparan sulphate molecules, suggesting a complex interplay of GAG-binding proteins or receptors and GAG molecules in early cell recognition events with this pathogenic prokaryotic intracellular organism [35]. It is likely that this is followed by interaction with other eukaryotic cell receptors, as proposed for heparan sulphate-binding viruses like herpes simplex, which may involve secondary binding to fibroblast growth factor receptors to trigger cell uptake and intracellular multiplication [2, 58].

Apart from mediating epithelial adhesion, the 28-kDa HEBP of *M. tuberculosis* and *M. bovis* mediates mycobacterial aggregation which, *per se*, may promote virulence by formation of microcolonies *in vivo* at the site of adhesion [18].

Possible role of glycosaminoglycan binding in intracellular survival

Survival and multiplication of intracellular microorganisms in macrophages and other eukaryotic cells is vital in the pathogenesis of infections caused by such microbes. It has been suggested that GAG-binding surface molecules confer the resistance to phagocytosis seen with *L. monocytogenes*, *H. pylori* and *N. gonorrhoeae* [19, 20, 59]. It is tempting to speculate that GAG binding may play an important role in intracellular survival in specific cells, such as macrophages, known to express mannose-sialoglycoconjugates and heparan sulphate [60]. Intracellular pathogens known to use heparan sulphate-like molecules in lectinophagocytosis are listed in Table 5 [12, 18, 19,

Table 5. Intracellular pathogens known to use heparan sulphate-like molecules in lectinophagocytosis

Organism	Reference
<i>Leishmania donovani</i>	[61]
<i>Trypanosoma cruzi</i>	[12]
<i>Plasmodium falciparum</i>	[29]
<i>Helicobacter pylori</i> *	[59]
<i>Listeria monocytogenes</i>	[19]
<i>Mycobacterium tuberculosis</i>	[18]
<i>Yersinia enterocolitica</i>	[62]

*Possibly not an intracellular organism.

29, 59, 61, 62]. Specific binding to macrophages has been observed in *H. pylori*, but the heparin-binding haemagglutinin of *M. tuberculosis* does not mediate binding to macrophages [18, 59]. Further studies on the possible role of GAG binding in intracellular multiplication of chlamydiae seem to be important to understand chronic intracellular survival of these pathogens, including *Ch. pneumoniae*, isolated from vascular endothelium of patients with atherosclerosis [63].

However, more in-vitro studies in serum-containing and serum-free systems with macrophages are necessary to define a possible interplay of GAG molecules with individual pathogens. Such studies will form a basis for deciding if GAG molecules such as heparin and various 'heparinoids' can be used in combination with other glycoconjugates to block uptake into macrophages, other professional phagocytes and other target cells in pathogenesis. Such cells include the human gastric epithelium for *H. pylori*, and the urogenital and respiratory tract epithelium for chlamydiae, *Bord. pertussis* and *Hae. influenzae*. Another genital tract pathogen, *N. gonorrhoeae*, has been found to interact with various cell types in heparin inhibition experiments [52]. *N. gonorrhoeae* expressing Opa A, C, F, G and H [20] absorb various exogenous polyanions such as nucleic acids and heparin. As heparin was found to increase the serum resistance of Opa⁺ compared with Opa⁻ gonococci, it has been proposed that cellular GAG molecules shed in genital fluid during infection may bind to Opa⁺ gonococci and protect them from phagocytosis, in the same way that sialylated capsules can protect *E. coli* K1 strains from phagocytosis in neonatal septicaemia and sialylated lipopolysaccharides can protect gonococci from phagocytosis [52].

Other biological effects

A number of adhesins to epithelial cells have been identified in *Yersinia enterocolitica*, including YadA, Ail and Inv [64]. The Yop virulon is a plasmid-encoded virulence apparatus which confers on *Yersinia* the ability to secrete a number of proteins that inhibit host defence mechanisms and enhance intracellular survival [65]. LcrG has been identified as a regulatory

component of Yop. LcrG has been shown to bind heparin and dextran sulphate, but not other GAG molecules, and this triggers translocation of Yop effectors inside eukaryotic cells [62]. Presumably, *Yersinia* cells can utilise heparan sulphate exposed on eukaryotic cells for translocation. Moreover, heparin binding may also interfere with complement and vitronectin binding, and modulate microbe–phagocyte interactions as well as binding to other cells, as shown for *C. albicans* [26], *Pneumocystis carinii* [66] and coagulase-negative staphylococci [67]. Epithelial cells undergo rapid apoptosis on loss of contact with the underlying matrix. As several ECM proteins have heparin-binding domains, it is possible that expression of heparin binding by microbes may enhance apoptosis by interfering with the matrix–epithelium contact.

Whether GAG binding may promote invasion of eukaryotic cells by microbes has been a matter of controversy. For herpes simplex, gpB binding of heparan sulphate appears to mediate adhesion, as well as virus–cell fusion and syncytium formation [68], and the cell surface protein ActA of *L. monocytogenes* mediates both adhesion to professional and non-professional phagocytes and internalisation by invasion through the basolateral surface of epithelial cells [69]. For other microbes, it may be more appropriate to refer to GAG adhesion as an initial, sometimes irreversible, adhesion leading to tight adherence, internalisation and inflammatory responses which may be mediated by activation of other receptor molecules [70].

At least three growth factors require heparin for activation. These are acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF). The finding that *S. aureus* and coagulase-negative staphylococci, as well as *H. pylori*, can bind bFGF and PDGF specifically [71, 72] may indicate that binding of heparin or growth factors by pathogens can interfere with wound healing in various organs. In addition, it has been suggested that heparan sulphate–growth factor interactions target *Pseudomonas aeruginosa* exotoxin to susceptible cells [73].

It can be speculated that GAG binding of rather low specificity occurs with surface proteins expressing basic amino acid-rich surface domains, followed by interactions with various acidic molecules in body secretions and serum, and that this may be important in creating ‘disguised’ organisms. Furthermore, surface domains may express proteins rich in hydrophobic domains (‘hydrophobins’) of importance for the pathogen to evade a strong immune response [74]. Thus, studies on gonococci, *H. pylori* and other GAG-binding pathogens seem to be of great importance because GAGBPs, as mucosal epithelium adhesins, are vaccine antigen candidates, a concept that would also target molecules besides various surface lectins and

ECM-binding proteins to create an anti-adhesion approach for prevention and treatment of infections by a ‘non-antibiotic’ strategy. Preliminary studies involving the use of modified heparin molecules and specific sialoglycoconjugates to block *H. pylori* GAGBPs and SALs in a BALB/c mouse and a primate model have been promising (X. Wang and T. Wadström, unpublished results; D. Zopf, personal communication).

Despite the fact that early studies showed that binding of heparin to the gp120 viral envelope protein of the human immunodeficiency virus inhibited viral entry into cells, syncytium formation, cytopathic effect, reverse transcriptase and binding to specific basic amino acid-rich domains of the Tat protein involved in attachment of the Tat protein to DNA and RNA [75], no systematic approach has been developed to design GAG-like molecules for use as anti-HIV drugs. However, a study by Howell *et al.* [31] with low mol. wt (LMW) commercial heparin (intended for conventional anticoagulant therapy) showed promising therapeutic results with few side-effects when used for self-administration subcutaneously twice daily in a pilot study. It was suggested that LMW heparin may well show synergy when used with other anti-retrovirus therapies.

Standard commercial heparin and LMW heparin (Fragmin) were shown to disrupt rosette formation between *P. falciparum* and both infected and non-infected red blood cells from patients with cerebral malaria [29]. A heparin fraction with low affinity for antithrombin III was equally as effective in disrupting such malaria parasite-induced cell rosettes. These findings suggest that GAG molecules may be true cell receptors on endothelial cells in the brain and on specific cells in other organs such as the liver and placenta. Other investigators have confirmed this proposal and shown that chondroitin-4-sulphate is the natural receptor for *Plasmodium* spp. in the placenta, and is probably involved in transmission of the parasite from the mother to the fetus [17, 76].

Altogether, these findings suggest that modified LMW heparin and other GAG molecules, or heparinoids, are indeed candidates for future development of antibacterial, antiviral and antiparasitic drugs. Interestingly, chronic parasitic infections such as leishmaniasis and trypanosomiasis are caused by parasites that use GAG molecules during early or late stages of infections for cell entry and trans-cell ECM-like interactions. Are there reasons to delay these developments when there is a great need for development of new antimicrobial drugs? It is known that pentasaccharide sequences in heparin and other GAG molecules synthesised by capillary endothelia and other cells in various body tissues show significant heterogeneity, and that this affects binding to a great variety of heparin-binding proteins in serum and other body

tissues [77]. These heparin-binding proteins can be subdivided into five major groups: i.e., chemokines, cytokines, ECM proteins, heparin-dependent growth factors, growth factor-binding proteins and a number of enzymes such as superoxide dismutase (Table 6). Moreover, GAGs are potent inhibitors of leucocyte rolling in the order heparin–chondroitin-6-sulphate, heparan sulphate and chondroitin-4-sulphate [32, 78]. It has been shown that this phenomenon is affected by a higher sulphate content in heparin and the position of sulphate groups in GAG molecules. These molecules also interact with L selectin on leucocytes and bind these cells to the endothelium, while E selectins recognise sialylated tetrasaccharides, such as Lewis X [32, 78]. Interestingly, some partly desulphated heparin derivatives have been tested for their effect on leucocyte–cell adhesion, which was shown to differ for 2-O desulphated, 2-O N-desulphated and N-desulphated heparin, suggesting that overall sulphate content, as well as regional sulphate density or specific sulphate groups, affect binding properties. It seems most likely that studies with such modified heparin molecules will reveal interesting differences in binding to various microbial GAG-binding proteins. Further studies in this area are necessary before candidate drug molecules can be developed as antiviral and antimicrobial drugs. Such studies should involve proteoglycans derived from target organs in specific situations in view of reports that *B. burgdorferi* may use decorin as a specific target molecule in joints in the development of arthritis in Lyme disease [21]. The observation that chondroitin-6-sulphate mediates adhesion of *P. falciparum*-infected erythrocytes to cerebral venular endothelium, but not heparin or heparan sulphate, suggests that a systematic study should involve various developmental stages of parasites other than *P. falciparum* [17]. The same is most likely true for intracellular pathogens such as *M. tuberculosis* and the various pathogenic *Chlamydia* spp. This is also of great importance in the development of GAG-based therapy to prevent tumour metastasis, where heparin and heparin-degrading enzyme inhibitors may inhibit tumour growth and tumour spread.

Table 6. Examples of heparin-binding proteins

Type of molecule	Heparin-binding proteins
Growth factors	Fibroblast, epithelial and endothelial growth factors, platelet-derived growth factor, TGF β
Growth factor-binding proteins	Follistatin
Cytokines	TNF α , IL-3, IL-8, IFN- γ , GM-CSF
Chemokines	RANTES, macrophage inflammatory protein 1
Extracellular matrix proteins	Fibronectin, laminin, thrombospondin, collagens, vitronectin
Enzymes	Cathepsin G, elastase, superoxide dismutase

The finding that a number of GAG- and ECM-binding micro-organisms, such as group A streptococci, *S. aureus*, *E. coli*, *B. burgdorferi* and *H. pylori*, bind and activate tissue and ECM-associated plasminogen to proteolytically active plasmin [79–83] suggests that several analogies may exist between (i) tumour growth and spread, and (ii) interplay between GAG binding and GAG and ECM degradation by specific enzymes involved in normal tissue regeneration and repair. A correlation between basement membrane heparan sulphate and normal epidermal proliferation in wounds has been suggested [84]. GAG-binding microbes can down-regulate tissue repair efficiently by destabilising heparin-dependent growth factors, as proposed for chronic herpes simplex mucosal infections and in *H. pylori* chronic type B gastritis and peptic ulcer disease [72]. Finally, GAG molecules are associated with cell release and ECM association of various chemokines and cytokines such as interleukin-8 (IL-8) and RANTES (Table 6), suggesting a role for GAG-like molecules in allergy and chronic mucosal surface inflammation in various organs. This is of relevance for *H. pylori*-induced type B gastritis, ulcerative colitis and other forms of inflammatory bowel disease. Reports of positive results with local heparin treatment on the healing process in the rectal mucosa of patients with ulcerative colitis suggest that further studies are warranted on the role of tissue GAG molecules in the normal gastrointestinal tract and inflamed mucosa [85]. Similarly, in the oral cavity, microbes involved in chronic periodontal disease, such as *Prevotella* spp. and *Porphyromonas gingivalis*, have been shown to bind specific ECM molecules, but have not yet been investigated for GAG-binding properties [86]. Reduced expression of heparan sulphate and syndecan-1 has been observed in inflamed human periodontium [87], which may suggest degradation by adhering micro-organisms. If so, this may be an important area for future prophylaxis. It is known that specific binding sequences of microbial lectins and, probably, GAG-binding proteins are usually well-preserved genetically compared with other parts of these ligands. It is probable that genetic characterisation of GAGBPs will define GAG-binding peptides which can also be used as tests for anti-adhesion and anti-infectious effects, analogous to those seen with a Fn 45-kDa fragment (Telios, San Diego, CA, USA) and specific binding domain sequences of staphylococcal FNBP, used to block *C. albicans* and *S. aureus* infections, respectively.

Conclusions

The data showing that a number of microbes may use GAG-binding proteins as adhesins, and the further involvement of GAG molecules during subsequent stages of the infectious process, make GAG molecules promising therapeutic agents. Studies with a *Bord. pertussis* vaccine based on the fimbrial haemagglutinin

(FHA) [88, 89] allow the proposal that GAG-binding proteins are suitable candidates to be incorporated in future vaccines against a number of microbial pathogens, such as chlamydiae, *H. pylori*, *M. tuberculosis*, malaria and *Leishmania* parasites, as well as viruses such as herpes simplex, CMV and HIV. The use of heparin or other GAG molecules to block adhesion is also attractive, as these substances are produced naturally by the human body. Therefore, allergic reactions and induction of antibodies are rare, although the latter has been reported in patients with renal failure [90].

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