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Host Cell Heparan Sulfate Proteoglycans Mediate Attachment and Entry of *Listeria monocytogenes*, and the Listerial Surface Protein ActA Is Involved in Heparan Sulfate Receptor Recognition

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The mechanisms by which the intracellular pathogen *Listeria monocytogenes* interacts with the host cell surface remain largely unknown. In this study, we investigated the role of heparan sulfate proteoglycans (HSPG) in listerial infection. Pretreatment of bacteria with heparin or heparan sulfate (HS), but not with other glycosaminoglycans, inhibited attachment and subsequent uptake by IC-21 murine macrophages and CHO epithelial-like cells. Specific removal of HS from target cells with heparinase III significantly impaired listerial adhesion and invasion. Mutant CHO cells deficient in HS synthesis bound and internalized significantly fewer bacteria than wild-type cells did. Pretreatment of target cells with the HS-binding proteins fibronectin and platelet factor 4, or with heparinase III, impaired listerial infectivity only in those cells expressing HS. Moreover, a synthetic peptide corresponding to the HS-binding ligand in *Plasmodium falciparum* circumsporozoite protein (pepPf1) inhibited listerial attachment to IC-21 and CHO cells. A motif very similar to the HS-binding site of pepPf1 was found in the N-terminal region of ActA, the *L. monocytogenes* surface protein responsible for actin-based bacterial motility and cell-to-cell spread. In the same region of ActA, several clusters of positively charged amino acids which could function as HS-binding domains were identified. An ActA-deficient mutant was significantly impaired in attachment and entry due to altered HS recognition functions. This work shows that specific interaction with an HSPG receptor present on the surface of both professional and nonprofessional phagocytes is involved in *L. monocytogenes* cytoadhesion and invasion and strongly suggests that the bacterial surface protein ActA may be a ligand mediating HSPG receptor recognition.

The gram-positive bacterium *Listeria monocytogenes* is the causative agent of listeriosis, an opportunistic infectious disease affecting humans and a wide range of animal species, including mammals, birds, and other vertebrates. Listeriosis manifests mainly as central nervous system infections, abortions, stillbirths, and septicemias. There are also rarer forms of listeriosis affecting the skin, joints, heart, lungs, pleura, intestine, or peritoneum (18, 60), indicating that *L. monocytogenes* holds the potential to infect multiple tissues. *L. monocytogenes* is a food-borne pathogen which gains access into the host by crossing the intestinal barrier (55). It then translocates to the mesenteric lymph nodes and disseminates to the spleen and liver. In the latter organ, bacteria multiply within hepatocytes, forming typical discrete infectious foci (5, 61). These early stages of listeriosis are mainly subclinical. If the bacteria are not cleared from primary target organs by the host immune cells (38), it is believed that they are transported by the bloodstream to secondary target organs (the central nervous system or the placenta and fetus), causing the characteristic clinical picture of listeriosis.

L. monocytogenes is a model facultative intracellular pathogen able to survive and multiply within macrophages (6, 61). It can also invade a wide range of normally nonphagocytic cell types, including enterocytes, hepatocytes, fibroblasts, and dendritic and endothelial cells, by inducing its own internalization (14, 25, 31, 42, 74). *L. monocytogenes* is well adapted to the intracellular niche due to a variety of features: it produces membrane-active exoproteins that mediate phagosomal disruption and bacterial escape to the cytosol; it has regulatory mechanisms for the preferential expression of bacterial genes in infected mammalian cells; and one of its surface proteins, ActA, mediates migration inside and between cells by exploiting the eukaryotic host cell cytoskeletal machinery (reviewed in references 43, 54, and 61).

Microbial attachment to host cells and tissues is the first step of the infectious process and cell invasion by intracellular pathogens (20). There is increasing evidence that intracellular pathogenic bacteria specifically recognize surface structures through receptor-ligand interactions (17, 19, 73). Involvement of C3bi (13) and C1q (1) complement receptors in *L. monocytogenes* uptake by phagocytic cells has been documented. *L. monocytogenes* can also be efficiently internalized in the absence of serum by various nonprofessional phagocytes, indicating that nonopsonic receptor-ligand interactions also participate in host cell recognition by *Listeria*. The listerial surface

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proteins InlA (or internalin) and InlB have been shown to mediate penetration into epithelial cells and hepatocytes, both in vitro and in vivo, and to have a role in cell tropism (11, 24, 26, 27). A receptor for internalin has been recently identified in E-cadherin (48), a cell-cell adhesion molecule present on basolateral surfaces of epithelial cells. Although InlA and InlB appear to play a significant role in listerial entry into host cells, *inlAB* mutants are still able to penetrate cells both in vitro and in vivo (11, 26, 48), which argues in favor of the existence of other invasion systems in *L. monocytogenes.*

L. monocytogenes has a broad pathogenic spectrum, and it can colonize and invade different animal tissues and target cells in numerous host species. Thus, adhesion should involve the recognition of a ubiquitous, surface-exposed host molecule. Besides extracellular matrix (ECM) proteins such as fibronectin, collagen, and laminin, which are known targets of bacterial cytoadhesion (16, 19, 73), glycosaminoglycans (GAG), also major ECM macromolecules, may play a role in the pathogenesis of infectious processes. Heparan sulfate (HS) is a major GAG component of proteoglycans. HS proteoglycans (HSPG) are integral components of plasma membranes and are ubiquitously distributed among cell populations of animal tissues, where they are thought to be involved in cell-cell and cell-ECM interactions. The structural diversity of HS chains offers many possibilities for biological recognition as revealed by the capacity of HSPG to interact with a variety of molecules, including growth factors, cytokines, enzymes, and ECM proteins (33, 37, 56, 75). These features of HSPG make them potential targets for microbial adhesion. Accordingly, they are being implicated increasingly as host cell receptors for a variety of microbial pathogens, including bacteria (32, 36, 70), protozoa (21, 46, 51, 52), and viruses (49, 62). We therefore investigated whether HSPG are involved in the pathogenesis of *L. monocytogenes* infection. Here we show that listerial interaction with the surface of host cells involves specific recognition of an HSPG receptor. We also report evidence that the listerial surface protein ActA, a major virulence factor primarily involved in actin-based intracellular motility, is a bacterial ligand in the recognition of the HSPG receptors.

MATERIALS AND METHODS

Media and buffers. The following media were used for cell cultures: RPMI 1640 (R0 medium); R0 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 50 μg of gentamicin per ml (R10 medium); Ham's F-12 medium (H medium); and H medium supplemented with 10% FCS and 0.1 mg of vancomycin per ml (H10 medium). Buffers used included Hanks' balanced salt solution (HBSS), Tris-buffered saline (TBS; 130 mM NaCl, 50 mM Tris [pH 7.4]), and phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA), 0.01 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, and 1 μg of leupeptin per ml (PBS-GAG). Blocking solutions included 10% FCS in HBSS (HBSS-FCS) and 1% BSA in TBS (TBS-BSA). All media and buffers were confirmed to be endotoxin free (<0.01 ng/ml) by a chromogenic *Limulus* amebocyte lysate microassay (Whittaker M. A. Bioproducts, Walkersville, Md.). Brain heart infusion (Difco Laboratories, Detroit, Mich.) broth or agar was used to culture bacteria; it was supplemented with 5 μg of erythromycin per ml for mutant strains to preserve the stability of the corresponding phenotypes.

Cell lines and bacterial strains. The murine macrophage-like cell line IC-21 was obtained from the American Type Culture Collection (ATCC TIB 186). IC-21 cells were maintained in R10 medium. Wild-type CHO-K1 cells and the GAG-deficient mutants CHO-745, CHO-761, and CHO-677 (15) were from J. Esko (University of Alabama, Birmingham). They were cultured in H10 medium.

L. monocytogenes L028, a clinical isolate of serovar 1/2c (40, 71), was used as the wild type in all experiments. The following L028 mutants were also used: LUT-12, deficient in ActA expression, is an *actA* insertion mutant obtained by transposon mutagenesis with a Tn*917* derivative (40); *hly*::Tn*917* is a nonhemolytic, Tn*917*-induced *hly* insertion mutant from L028 (from J. C. Pérez-Díaz, Madrid, Spain); and *plcB*, ORFX/Y, and ORFZ mutants are obtained by disruption of the corresponding genes by homologous recombination with the thermosensitive plasmid pHV1248ΔTn*10* carrying target DNA sequences (40, 71). Bacteria were grown at 37°C. They were stored at −70°C in PBS with 20% (vol/vol) glycerol until used. Bacterial concentrations were calculated by both plate counting and densitometry (A_{660}).

General reagents. Aprotinin, heparin, HS, chondroitin sulfate, keratan sulfate, dermatan sulfate, fucoidan, hyaluronic acid, colominic acid, fibronectin, platelet factor 4, fluorescein isothiocyanate (FITC; isomer I), and the enzymes hyaluronidase (EC 3.2.1.35), chondroitinase ABC (chondroitin ABC lyase; EC 4.2.2.4), heparinase I (heparin lyase I; EC 4.2.2.7), and heparinase III (heparin lyase III; EC 4.2.2.8) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Phenylmethylsulfonyl fluoride and leupeptin were from Calbiochem (San Diego, Calif.), and *p*-nitrophenyl β-D-xylopyranoside (PNP-xyloside) was from Boehringer GmbH (Mannheim, Germany). [^{3}H]thymidine was from Amersham.

Peptides. The following synthetic peptides were used: pepPf1 (4), with the sequence PCSVTCGNGIQVRIKPGSAN corresponding to that of region II from the *Plasmodium falciparum* circumsporozoite (CS) protein; BLA(63-79), with the sequence DDQNPHSSNICNISCDK corresponding to that of bovine α-lactalbumin; and the fibronectin-derived RGD peptide, with the sequence RGDSPASSKP. The pepPf1 was used in aggregated form for maximal activity (63). For this, prior to utilization, the peptide was oxidized by stirring overnight at room temperature in Tris (pH 8). To verify the inhibitory activity of the peptide, disulfide-linked aggregates were separated from monomers by molecular sieving through a Sephadex superfine G-50 (Pharmacia) column equilibrated in TBS (pH 6.45). Only fractions corresponding to oligomeric forms inhibited listerial binding.

Measurement of bacterial binding to IC-21 macrophages. To evaluate bacterial binding to IC-21 cells, which adhere poorly to plasticware, a flow cytometric assay was used. Bacteria were suspended in 600 μl of a 0.1-mg/ml concentration of FITC in PBS and incubated with shaking for 2 h at 37°C in the dark. The bacteria were then thoroughly washed until the supernatant was devoid of any free FITC as measured spectrophotometrically against a PBS standard. FITC-labelled *L. monocytogenes* (10^8/ml) was added to 1 to 4% paraformaldehyde-fixed IC-21 cells in suspension (10^6/ml), and the resulting mixtures were incubated at 4°C for 30 to 60 min. To determine the effect of GAG lyases on *L. monocytogenes* binding, cell monolayers were treated at 37°C for 45 min with 0.5 to 1 IU of the corresponding enzyme per ml in PBS-GAG. Treated cells were washed three times with ice-cold PBS prior to the binding assay. For binding inhibition assays using synthetic peptides, fixed IC-21 cells were washed three times with TBS and then blocked with TBS-BSA for 2 h at 37°C. Peptides were incubated with IC-21 cells at concentrations ranging from 0 to 500 μg/ml in TBS. All incubations with FITC-labelled *L. monocytogenes* were done in the dark. Target cells were then washed, and bound bacteria were determined by flow cytometry with a FACScan flow cytometer (Becton Dickinson). A total of 10,000 events per sample were collected by 4-$\log_{10}$ amplification. Data were analyzed on a HP9000 series model 310 computer, and histograms were generated with LYSIS II software.

Measurement of bacterial binding to CHO cells. CHO-K1 cells and their mutants attach firmly to tissue culture plates. To avoid the need for a trypsinization step which could remove membrane structures involved in bacterial attachment, a binding assay using radiolabelled *L. monocytogenes* was used. Bacteria were labelled by adding 740 KBq of [^{3}H]thymidine per ml to 10 ml of a mid-log-phase *L. monocytogenes* culture. Confluent monolayers of CHO cells (2×10^5 cells/well) in a polyvinyl 96-well cell culture plate (Falcon; Becton Dickinson) were fixed with 1% paraformaldehyde and treated with HBSS-FCS to block nonspecific binding sites. CHO cells were then incubated with radiolabelled bacteria (10^4 cpm) in 0.1 ml of PBS in the presence or absence of different GAG (60 min at 0°C) or synthetic peptides (overnight at 4°C). After washing, the wells were cut and the radioactivity associated to adherent CHO cells was measured in a scintillation counter.

Uptake assays. Confluent IC-21 or CHO monolayers, prepared by seeding 96-well cell culture plates (Costar) with 100 μl of cell suspension per well (usually, 1×10^5 IC-21 and 4×10^5 CHO cells/well), were used for uptake assays. Bacterial internalization was measured by a previously described assay (1). To test the effect of different GAG on *L. monocytogenes* uptake, 2×10^7 bacteria/ml were preincubated for 15 min at room temperature with each of a series of concentrations (0 to 500 μg/ml) of each GAG in PBS; 100 μl of each mixture was then added to each well to inoculate the target cells. In some experiments, the effect of GAG lyases on *L. monocytogenes* uptake by target cells was studied. Host cell monolayers or bacteria (2×10^7 bacteria/ml of suspension) were treated at 37°C for 45 min with 0.5 to 1 IU of the corresponding enzyme per ml in PBS-GAG. Treated cells or bacteria were washed three times with PBS prior to the uptake assay. GAG lyase treatment was proved not to affect bacterial viability and not to be toxic for target cells. For inhibition assays with HS-binding proteins, target cells were treated with fibronectin (500 μg/ml) or platelet factor 4 (2 μg/ml) at 4°C for 60 min in PBS, washed three times with ice-cold PBS, and then infected with bacteria. To study the effect of PNP-xyloside, IC-21 cells were pretreated with 10 mM of the drug for 60 min before the uptake assay. In all cases, uptake determinations were done in triplicate.

RESULTS

Inhibition of *L. monocytogenes* attachment and uptake by different GAG. Various purified soluble GAG were incubated

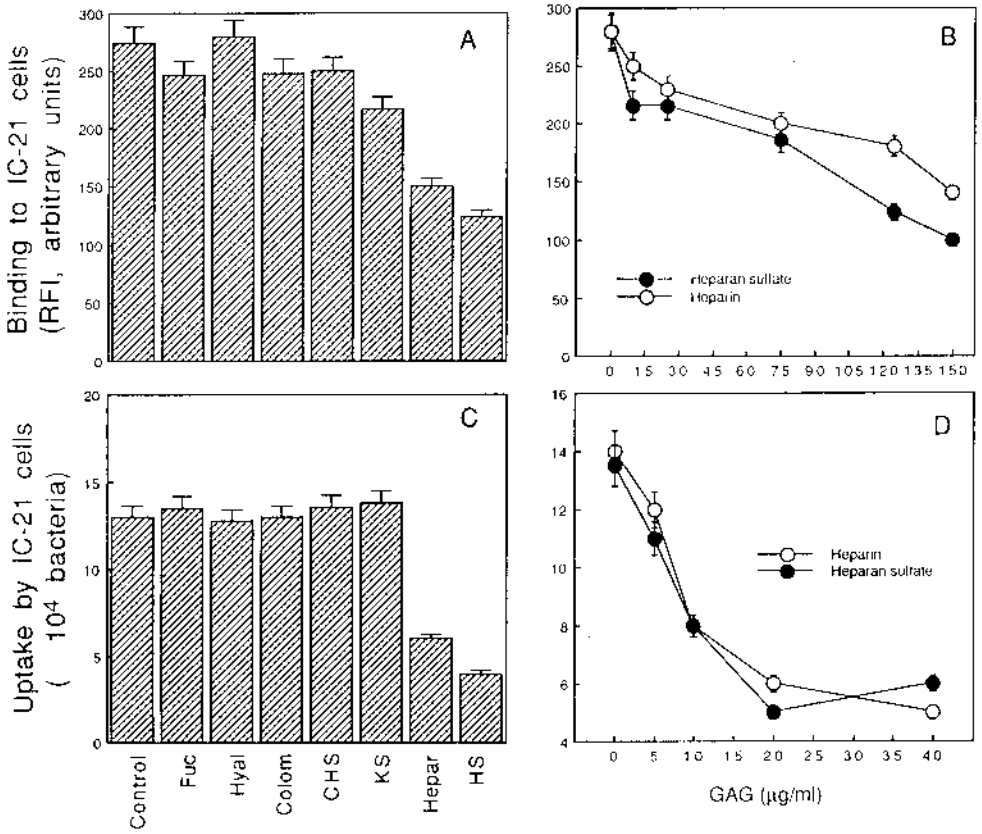


FIG. 1. Inhibition of *L. monocytogenes* binding and uptake by heparin and HS in IC-21 macrophage-like cells. (A and B) Binding inhibition assays. *p*-Formaldehyde-fixed IC-21 cells (10^6/ml) were incubated with 10^8 FITC-labelled bacteria/ml previously incubated with the indicated GAG (Fuc, fucoidan sulfate; Hyal, hyaluronic acid; Colom, colominic acid; CHS, chondroitin sulfate; KS, keratan sulfate; Hepar, heparin). Results are expressed in arbitrary units of the relative fluorescence intensity (RFI). (C and D) Uptake inhibition assays. Cells were infected at a ratio of 1:10 with bacteria previously incubated for 15 min at room temperature with 500 μg of the indicated GAG per ml (defined above) (C) or with one of a series of amounts of heparin or HS (D). Results are mean CFU ± standard deviation of three independent determinations.

with bacteria, and the inhibition of attachment to IC-21 murine macrophage-like cells was investigated. Of all the glycoconjugates tested, only heparin and HS significantly inhibited (46 to 57%) bacterial binding to IC-21 cells (Fig. 1A). Inhibition by heparin and HS was dose dependent (Fig. 1B). Similar results were obtained when uptake was measured (49 to 69% inhibition) (Fig. 1C and D). Despite being similar in sulfation degree to heparin and HS, fucoidan, chondroitin sulfate, and keratan sulfate did not inhibit attachment or uptake. Thus, the inhibition observed was dependent on the particular structural features of heparin and HS.

Attachment and uptake inhibition experiments were also performed with the normally nonphagocytic, epithelial-like, hamster-derived cell line CHO-K1. The results were comparable to those obtained with IC-21 macrophages (Fig. 2), indicating that HS seems to compete with a host cell surface structure, presumably an HSPG which would be present in professional and nonprofessional phagocytes.

Involvement of an HSPG in *L. monocytogenes* interaction with host cells. Various GAG lyases were also used to analyze the role of HS in the interaction of *L. monocytogenes* with host cells. Both bacteria and IC-21 cells were independently treated with chondroitinase ABC, which specifically removes chondroitin sulfate and dermatan sulfate from cell surfaces, and heparinase III, which specifically hydrolyzes the glycosidic linkage present in HS. Only enzyme treatment of host cells with heparinase III significantly affected *L. monocytogenes* attachment and uptake (Fig. 3). Treatment of bacteria with GAG lyases did not affect bacterial binding or uptake (data not shown). These results implicate HS in the host cell cytoplasmic membranes in *L. monocytogenes* attachment and uptake by IC-21 macrophages.

To confirm that a surface-exposed HSPG is required for cell infection by *L. monocytogenes*, well-characterized CHO-K1 mutants with defects in various aspects of GAG biosynthesis were used. These CHO mutants have been used extensively as tools to investigate the involvement of HSPG in various cell functions. CHO-745 and CHO-761 mutants lack, respectively, the xylosyltransferase and galactosyltransferase that mediate GAG synthesis and make little if any GAG. The CHO-677 mutant is deficient in HS synthesis but produces normal levels of chondroitin sulfate (15). CHO-745, CHO-761, and CHO-677 bound (Fig. 4A) and internalized (Fig. 4B) significantly fewer bacteria than the CHO wild-type cells (i.e., CHO-K1; binding and uptake inhibition levels were 39.9 to 47.5% and 44.6 to 53.7%, respectively). Treatment with the HS-specific lyase heparinase III, but not with chondroitinase ABC, had an inhibitory effect on *L. monocytogenes* interaction with CHO-K1 cells, which was not observed with the CHO mutants (Fig. 4C and D).

To further demonstrate that chondroitin sulfate plays no major role in *L. monocytogenes*-host cell interaction, IC-21 cells were treated with 10 mM PNP-xyloside (a competitive substrate for chondroitin sulfate synthesis) and then infected.

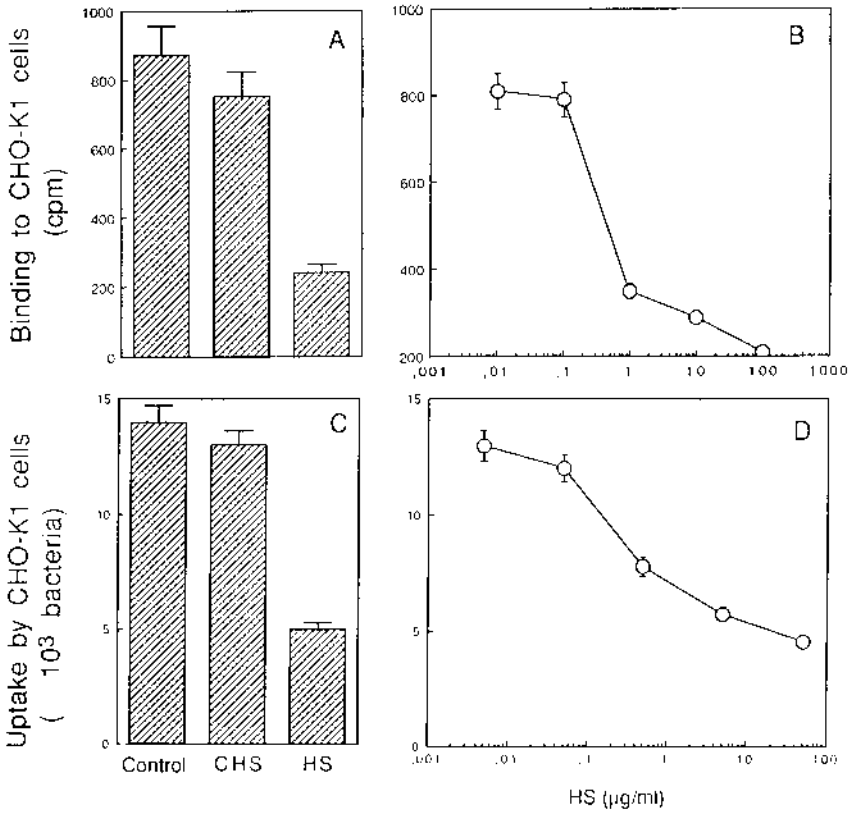


FIG. 2. Inhibition of *L. monocytogenes* binding and uptake by HS in CHO-K1 cells. Bacteria were preincubated with CHS (at 500 μg/ml) or HS (at concentrations of 100 [A] and 50 [C] μg/ml and as shown in the figure [B and D]) prior to being added to target cells. (A and B) Binding inhibition assays. Bacteria were labelled with [^{3}H]thymidine (10^5 cpm/ml), and results are expressed as mean counts per minute ± standard deviation of three independent experiments. (C and D) Uptake inhibition assays. Uptake was measured as described for IC-21 cells (see Fig. 1 legend).

There was no difference between the numbers of bacteria taken up by treated (25×10^4 bacteria/well) and untreated (26×10^4 bacteria/well) cells, indicating that chondroitin sulfate is not involved in listerial cytoadhesion and invasion.

The role of HS in *L. monocytogenes* recognition of target cells was also explored by using HS-binding ligand analogs. If HS is a receptor for *L. monocytogenes*, preincubation of target cells with HS ligands would interfere with *L. monocytogenes* infectivity. Fibronectin and platelet factor 4 are polypeptides which bind with high specificity to HSPG (37). Preincubation of the various CHO cell lines with these ligand analogs had an inhibitory effect only in those cells synthesizing HS (wild-type CHO-K1 cells) (Table 1).

Thus, an HS-containing structure, probably an HSPG on the surface of eukaryotic cells, seems to act as a receptor for *L. monocytogenes.*

Inhibition of *L. monocytogenes* attachment by a malarial HS-binding synthetic peptide. The microbial ligands involved

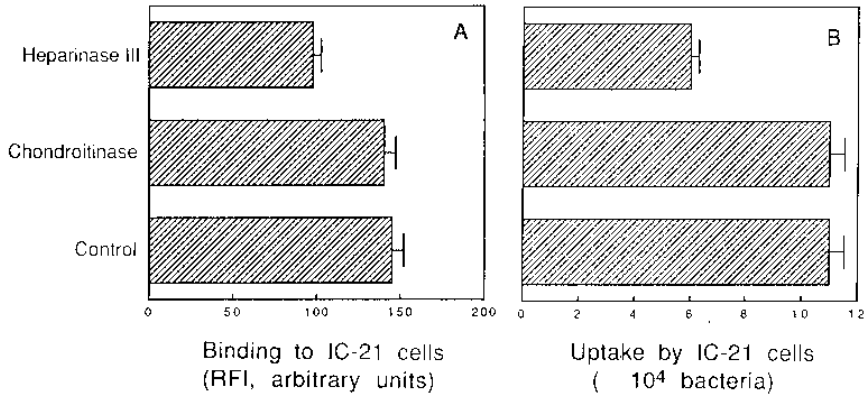


FIG. 3. Effect of various GAG lyases on *L. monocytogenes* binding (A) and uptake (B) by IC-21 cells. (A) Fixed cells were pretreated with 1 IU of the indicated GAG lyases per ml; results are expressed in arbitrary units of the relative fluorescence intensity (RFI). (B) IC-21 macrophages (10^5 cells/well) were treated with 0.5 IU of the indicated GAG lyases per ml and infected at a 1:10 multiplicity of infection. Results are expressed as mean CFU ± standard deviation of three determinations.

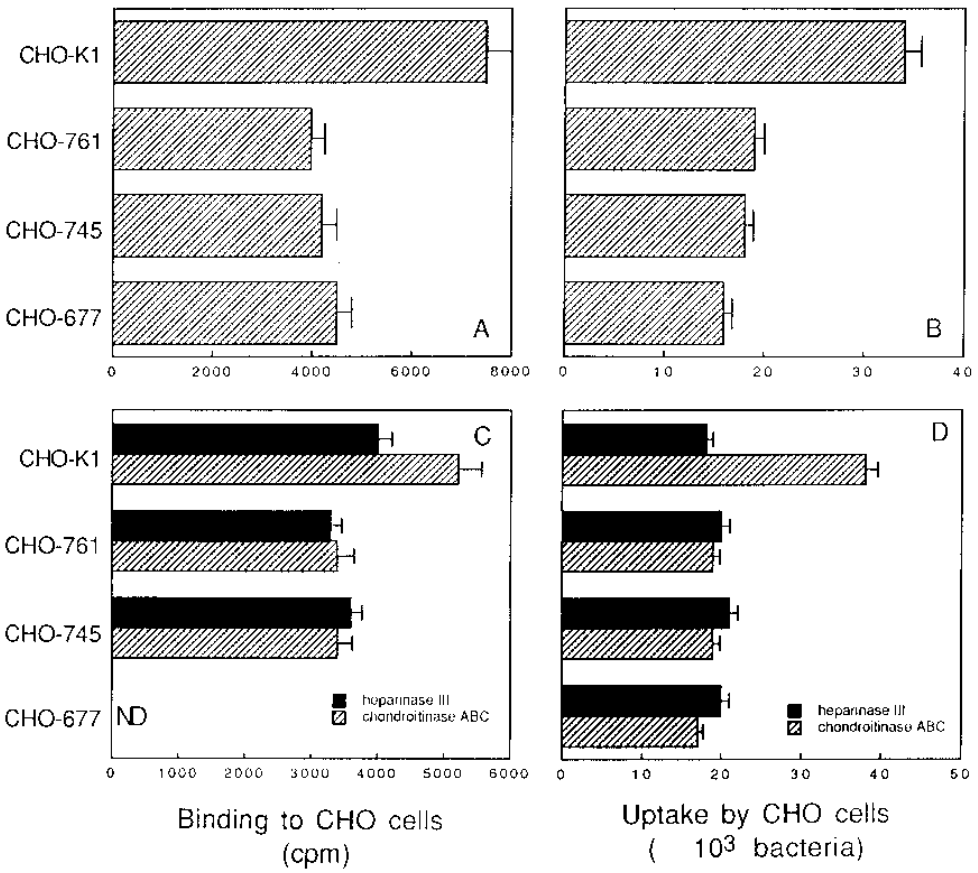


FIG. 4. Differential *L. monocytogenes* binding (A and C) and uptake (B and D) in wild-type and mutant CHO cells. For the experiments illustrated in panels C and D, cells were treated with heparinase III or chondroitinase ABC prior to the binding or uptake assay.

in HSPG attachment are presumably exposed at the surface. This is the case of the *P. falciparum* CS protein, which coats the parasite surface and is responsible for attachment to the hepatocyte basolateral membranes through HSPG-specific receptor binding (21, 52). The CS domain involved in HSPG binding has been recently identified as a motif in region II of the protein (63). A synthetic peptide representing the cell adhesive motif of region II, pepPf1, inhibits the interaction of CS protein with target cells and sporozoite invasion (4, 21, 63). Since pepPf1 is a ligand for HSPG receptor on target cells, it might have some inhibitory activity in our model if there was a common mechanism of recognition.

We tested labelled *L. monocytogenes* cells for attachment to fixed CHO-K1 and IC-21 cells which were pretreated with various amounts of the malarial peptide pepPf1 or an unrelated peptide, BLA(63-79) (see experimental procedures), as a control. Preincubation of cells with pepPf1 inhibited, in a dose-dependent manner, *L. monocytogenes* binding to target cells, whereas peptide BLA(63-79) did not affect listerial attachment (Fig. 5). These results demonstrate that the listerial ligand involved in HSPG binding, and the corresponding mechanism underlying such interaction, is similar to that of the malarial model.

Maximal inhibition was about 40% (Fig. 5), a percentage somewhat lower than that currently found in the malarial model with the same peptide, around 60 to 80% (4, 21, 52, 63). This moderate inhibition indicates that the mechanism underlying pepPf1 recognition is only partially responsible for *L. monocytogenes* interaction with target cells. Owing to the large number of structural possibilities of HSPG (multiplicity of sites and degrees of sulfation and variability in epimerization of hexuronate moieties and in core protein sequence), and since different molecular classes of HSPG are synthesized by different tissues (9, 33, 66), it can also reflect a lower degree of recognition specificity of the malarial synthetic peptide for the HSPG expressed by IC-21 and CHO target cells.

Identification of an *L. monocytogenes* ligand involved in HSPG binding. The structural features responsible for HSPG

TABLE 1. Effect of fibronectin (500 μg/ml) and platelet factor 4 (2 μg/ml) on *L. monocytogenes* interaction with CHO cells

Cell type	Effect of:					
	No treatment		Fibronectin		Platelet factor 4	
	Binding[a]	Uptake[b]	Binding	Uptake	Binding	Uptake
CHO-K1	1,283 ± 40	30 ± 0.8	725 ± 105	14 ± 3.2	483 ± 204	9 ± 0.1
CHO-761	966 ± 39	19 ± 3.6	796 ± 76	16 ± 2.1	ND[c]	15 ± 0.2
CHO-745	733 ± 50	16 ± 1.2	801 ± 64	18 ± 0.8	845 ± 166	15 ± 0.09
CHO-677	705 ± 27	13 ± 1.2	ND	14 ± 2.1	813 ± 289	13 ± 0.04

[a] Values are in counts per minute (mean ± standard deviation of triplicate experiments).
[b] Values are in CFU (10^3) (mean ± standard deviation of triplicate experiments).
[c] ND, not determined.

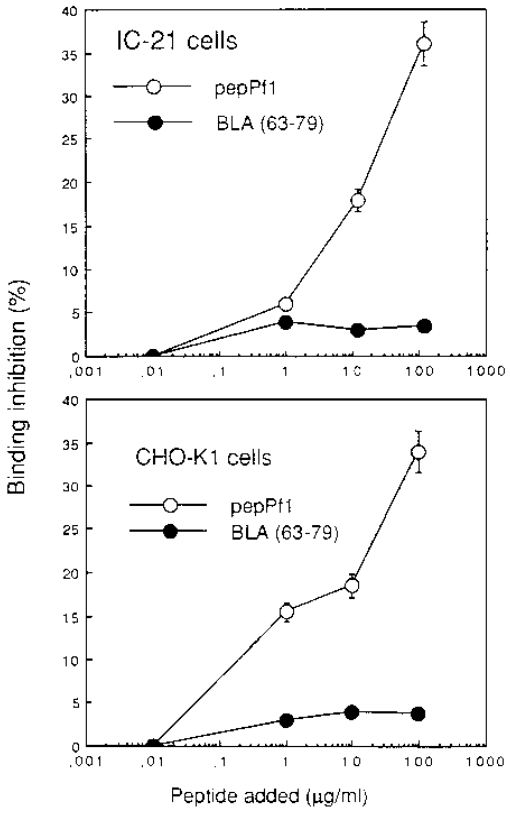


FIG. 5. Inhibition of *L. monocytogenes* attachment following treatment of target cells with the HS-binding malarial peptide pepPf1. IC-21 cells in suspension (2×10^6/ml) (upper panel) or confluent monolayers (4×10^5/well) of CHO-K1 cells (lower panel) were fixed with 1% *p*-formaldehyde, incubated with concentrations of peptide pepPf1 or BLA(63-79) (unrelated peptide used as control) ranging from 1 to 120 μg/ml, and exposed to FITC-labelled bacteria and [^{3}H]thymidine-labelled bacteria, respectively. Bound bacteria were determined by flow cytometry (IC-21 cells) or by scintillation counting (CHO-K1 cells), and data were expressed in arbitrary units of the mean relative fluorescence intensity or as mean counts per minute, respectively. Results are expressed as the percentage of inhibition with respect to the untreated control, calculated by the following formula: % inhibition = 100 − [(cells without peptide − cells with peptide)/(cells without peptide)].

interaction in region II of the malarial CS protein have been identified and consist of a triplet containing at least two basic amino acids [RX(K/R)], with neighboring interdispersed hydrophobic residues (63) (Fig. 6). Proteins with known heparin- or HS-binding activity contain clusters of different lengths of positively charged residues, in different combinations, which would mediate the establishment of ionic interactions with the sulfate groups of the acidic polysaccharide (37) (Fig. 6). It is therefore possible that similar structures were present in the *L. monocytogenes* ligand involved in HS interaction. Few *L. monocytogenes* surface proteins have been identified to date (12, 54). One of them, the actin polymerization protein ActA (40, 43, 71), has some characteristics consistent with it being involved in HS-mediated host-cell attachment. ActA is a surface protein which coats the bacterial cell; its predicted secondary structure is predominantly α-helical, and it is fixed to the bacterial surface through a highly hydrophobic, C-terminal membrane anchor (see Fig. 6); therefore, the protein could be conceived as a filamentous structure with its N terminus exposed above the cell envelope (40, 50, 71). At the N terminus of the mature ActA protein, there are three stretches of 40 to 54 amino acids containing an unusually high content (27%) of positively charged residues (K and R) (Fig. 6). These basic amino acids are mainly clustered and in close proximity to hydrophobic residues, a pattern strongly resembling the domains involved in HS recognition in HS-binding proteins (Fig. 6). Most interestingly, one of these clusters in ActA is within a stretch of 10 amino acids (98 to 107) whose sequence is almost identical to that of the pepPf1 domain involved in HS binding (Fig. 6).

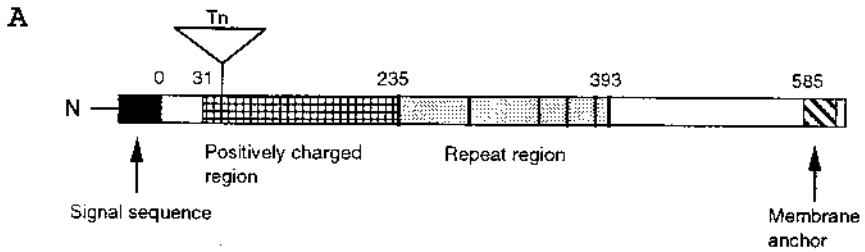
The *L. monocytogenes* ActA-deficient mutant LUT-12 was used to investigate the involvement of ActA in HS recognition. This mutant was constructed by transposon mutagenesis of *actA*, the structural gene for ActA, with a Tn*917-lac* derivative (40). As a result of *actA* disruption, LUT-12 is defective for actin polymerization and is totally avirulent. *actA* is part of an operon which includes other genes located downstream, i.e., *plcB* coding for a wide-substrate-range phospholipase C or lecithinase, involved in phagosome escape, and three additional open reading frames (ORFs) of unknown function (71). To control for the polar effect of transposon insertion on these downstream genes, three previously described mutants (*plcB*, ORFX/Y, and ORFZ mutants) (40, 71), obtained by homologous recombination with a thermosensitive plasmid bearing part of the target sequences, were also used.

The ActA mutant LUT-12 was significantly less efficient than the wild-type L028 strain in attaching to IC-21 and CHO-K1 cells (32 and 71% reduction in binding, respectively) (Fig. 7A and C). Similar results were obtained for uptake (30 to 51% and 57%, respectively) (Fig. 7B and D). The three other insertion mutants in the lecithinase operon exhibited the same pattern as the wild type did in IC-21 binding experiments, similar to the results obtained with a transposon-induced hemolysin-negative mutant of strain L028 which was also used as a control (data not shown). These data are strong evidence that the *actA* gene product is involved in listerial attachment to host cells.

To demonstrate that the impaired attachment shown by LUT-12 was due to the absence of HS recognition functions, the HS-deficient mutant CHO-761, CHO-745, and CHO-677 cells were used in binding and uptake assays with the mutant bacteria. LUT-12 attachment and invasion levels in all of the CHO cells were almost identical regardless of whether they expressed HS (Fig. 7C and D), consistent with the ActA mutant being affected in the expression of an HS ligand. It is interesting to note that wild-type bacteria infected the CHO mutants slightly better than LUT-12 did (Fig. 7C and D), implicating the listerial protein ActA in an HS-independent target cell recognition mechanism. ActA has an RGD motif (amino acids 231 to 233) (71) which could interact with the host cell surface via integrin receptor recognition, as has been reported in other bacterial models (16, 19). To evaluate the involvement of the RGD sequence in *L. monocytogenes* attachment, binding assays were performed with IC-21 cells in the presence and absence of a synthetic, fibronectin-derived RGD peptide, with and without pepPf1 coincubation. Consistent with previous observations (10), the RGD peptide had no detectable effect on binding efficiency, and coincubation with the RGD peptide and pepPf1 did not enhance binding inhibition (Table 2). Thus, the RGD sequence present in ActA does not seem to contribute to listerial attachment to target cells.

DISCUSSION

Listeriosis is a multisystemic infection in which listeriae have to cross several epithelial and endothelial barriers en route to their intracellular compartment in target organs. This implies the interaction with, and possibly the specific recognition of,



B

(31) RYETAREVSSRDIKELEKSNKVRNTNKADLI▼AMLKEKAEK
GPNINNNNSEQTENAAINEEASGAD
RPAIQVERRHPGLPSDSAAEIKKRRKA
IASSDSELESLTYPD
KPTKVNKKKVAK
ESVADASESDLDSSMQSADESSPQPL
KANQQPFFPKVPKKIKDAGKWVRDKIDENPEVKKAIVDKSAGLIDQLLTKKK (227)

C

ATh III (125-142)*	KLNCRLY	RKA	NKSSKLV
HC II (185-202)*	KLTHRLF	RRN	FGYTLRSV
Apo B (3144-3159)	SVKAQY	KKN	KHRHSI
Apo B (3352-3369)	YKLEGTTRLT	RKR	GLKLAT
Apo E (142-152)	RKL	RKR	LLR
EGF-like GF (28-35)	EHG	KRK	KK
EGF-like GF (36-43)	GKGLG	KKR	
EGF-like GF (44-52)	DPCL	RKY	KDF
ECGF/aFGF (90-102)	TYIS	KKH	AEKHWF
aFGF (124-138)	GL	KKN	GSCKRGP
bFGF (21-27)	ANY	KKP	K
FN (C-terminal region)	PPREVVP	RPR	PGV
FN (2035-2045)	ATPI	RHR	PRPY
FN (2145-2155)	KDQQ	RHK	VREE
PF4 (60-70)	PLY	KKI	IKKLLES
Th (229-241)	YTHVF	RLK	KWIQK
ThS (20-34)	ARKGSG	RRL	VKGPD
ThS (77-83)	RQM	KKT	R
VN (342-354)	RPSLA	KKQ	RFRHR
VN (352-369)	NRKGYRSQ	RGH	SPGR
vWF (565-580)	YIGL	KDR	KRPSELRRI
vWF (633-648)	NFVRYVQGL	KKK	KVIV
ActA (40-57)*	SRDIKELEKSN	KVR	NTNK
ActA (58-70)	ADLIAML	KEK	AEK
ActA (111-124)	DSAAEI	KKR	RKAIA
ActA (137-151)	DKPTKVN	KKK	VAKES
ActA (181-194)	PFFPKVF	KKI	KDAG
ActA (192-204)	DAGKW	RDK	IDEN
ActA (205-216)	PEV	KKA	IVDKSA
ActA (217-230)	GLIDQLLT	KKK	SEE
ActA (95-109)	DRP	AIQVERRH	PG	LP
PepPf1	PCSVTCGN	GIQV	RIK	PG	SAN

FIG. 6. Structure of ActA, sequence of its positively charged N-terminal region, and distribution of basic amino acids in ActA and in heparin-binding motifs of various proteins. (A) Schematic representation of the ActA protein. The position of Tn*917lac* insertion in the *actA* mutant LUT-12 is indicated (Tn). Numbers correspond to amino acid positions. (B) Amino acid sequence of the positively charged N-terminal region of ActA (cross-hatched bar in panel A). The segment 99-122 identified by Pistor et al. (53) as essential for actin accumulation and the motif 64-LKEK-67 similar to the actin-binding site of caldesmon (72) are underlined. The inverted triangle between amino acids 61 and 62 indicates the location of transposon insertion in the *actA* mutant LUT-12. Numbering is from the N terminus of the mature protein. (C) Alignment of putative HS-binding motifs in ActA with selected heparin-binding domains of GAG-binding proteins (AT III, antithrombin III; HC II, heparin cofactor II; Apo, apolipoprotein; EGF-like GF, epidermal growth factor-like growth factor; ECGF, endothelial cell growth factor; aFGF, acid fibroblast growth factor; bFGF, basic fibroblast growth factor; FN, fibronectin; PF4, platelet factor 4; Th, thrombin; ThS, thrombospondin; VN, vitronectin; vWF, von Willebrand factor) (sequences are from references 4, 34, 37, 65, and 71). It is important to note that in sulfated GAG-binding proteins, there is not a particular consensus sequence in heparin-binding domains except that they are rich in basic amino acids (usually clustered) and have interdispersed hydrophobic residues. Sequences marked with an asterisk are examples of α-helical stretches which could form cationic clusters on one side of the helix. PepPf1 is the *P. falciparum* synthetic peptide corresponding to the HS-binding ligand present in region II of the malarial CS protein (4). Amino acids shared by pepPf1 and ActA are boxed. The pepPf1 sequence is an evolutionarily conserved motif found in surface proteins of various malarial species, in *Eimeria tenella* and *Caenorhabditis elegans* proteins EtHL6 and UNC-5, respectively, and in a variety of mammalian proteins such as complement proteins C6 and C7, the neuronal adhesion protein F-spondin, and the heparin-binding proteins properdin and thrombospondin (30, 35, 57, 63). Basic residues in pepPf1-related sequences are clustered in a triplet with the consensus sequence R-X-B, where B denotes a basic residue (mostly R or K) and X denotes any amino acid (mostly basic, but I, S, T, G, Q, E are also found). In panels B and C, basic amino acids are in bold type.

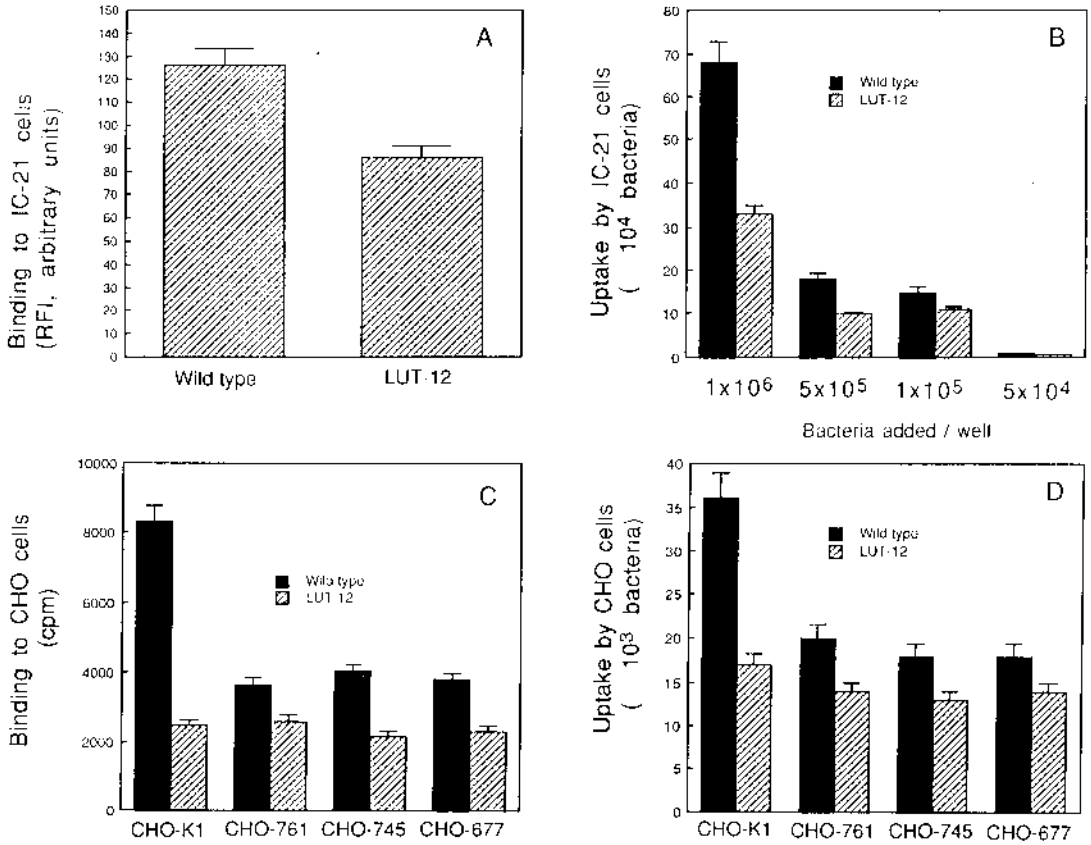


FIG. 7. Attachment and invasivity of the ActA-deficient *L. monocytogenes* mutant LUT-12 compared with that of the wild-type parent strain L028. Binding (A) and uptake (B) in IC-21 macrophages and binding (C) and uptake (D) in wild-type and mutant CHO epithelial-like cells are shown. Binding and uptake are significantly impaired in the ActA-deficient mutant.

one or more target structures, which may include components of the ECM of plasma and basement membranes and connective tissues. Here we report that an HSPG which is expressed on the surface of at least IC-21 macrophages and CHO epithelial-like cells acts as a receptor for *L. monocytogenes.*

TABLE 2. The RGD motif of ActA does not contribute to listerial binding to IC-21 cells

IC-21 cells incubated with:			*L. monocytogenes* binding[a] (% inhibition)
BLA(63-79) (μg/ml)	pepPf1 (μg/ml)	RGD peptide (μg/ml)	
0	0	0	521 (0)
10	0	0	497 (4.60)
50	0	0	499 (4.22)
100	0	0	577 (−10.74)
0	0	10	505 (3.07)
0	0	50	484 (7.10)
0	0	100	504 (3.26)
0	10	0	477 (8.44)
0	50	0	372 (28.59)
0	100	0	345 (33.78)
0	50	10	491 (5.7)
0	50	50	409 (21.4)
0	50	100	361 (30.71)

[a] Binding of FITC-labelled *L. monocytogenes* to fixed IC-21 cells, with or without preincubation with increasing amounts of the indicated peptides, was measured by flow cytometry (see Materials and Methods for details). Results are expressed as the relative fluorescence intensity in arbitrary units.

In our results, binding to host cells through recognition of the HSPG receptor is associated with the concomitant uptake of *L. monocytogenes* by both professional and nonprofessional phagocytes. Some HSPG are bound to cell membranes through a membrane-spanning core protein, such as syndecans, which are produced by most mammalian cells (2, 8). Syndecans colocalize with actin bundles at the cell surface and are thought to act as bridges between the ECM and the intracellular cytoskeleton (56, 75). *L. monocytogenes* binding to syndecan structures may thus transduce signals triggering local cytoskeletal rearrangements, resulting in phagocytosis. In epithelial confluent cells, syndecans are mostly expressed on basolateral membranes (37), and this is concordant with recent experimental evidence suggesting that *L. monocytogenes* invades epithelial confluent cells through the basolateral surface (26, 67). A syndecan-like HSPG has been recently found to be involved in invasion of host cells by another bacterial pathogen, *Neisseria gonorrhoeae* (70).

Other HSPG are covalently bound to the plasma membrane via glycosylphosphatidylinositol anchors (37). Interestingly, *L. monocytogenes* secretes a phosphatidylinositol-phospholipase C which is involved in virulence. Although it is thought to be primarily involved in phagosomal membrane disruption (64), the enzyme is able to cleave glycosylphosphatidylinositol anchors (28, 47) and thus might also participate in virulence by modulating the interaction of *L. monocytogenes* with target plasma membranes.

Fibronectin and platelet factor 4, both heparin-binding proteins, interfered with the listerial interaction with the host cell

surface only in those cells expressing HS (Table 1). An alternative system of GAG-mediated listerial attachment and invasion is thus possible, via receptors of heparin-binding proteins (e.g., the integrin receptors for fibronectin), by the use of secreted sulfated GAG (e.g., heparin) as bridging molecules. In addition, accumulation of a host-derived secreted sulfate-GAG over the listerial surface may also constitute a mechanism for evading the soluble bactericidal response of the immune system.

A key finding of this study is that the *P. falciparum* peptide pepPf1 (Fig. 6), corresponding to the malarial ligand responsible for CS binding to HSPG receptors on target plasma membranes (3, 4, 52), specifically and dose dependently inhibited attachment of *L. monocytogenes* to IC-21 and CHO cells (Fig. 5). The cross talk between pepPf1 and *L. monocytogenes* led us to look for a structural homolog in this bacterium. Interestingly, we found that the listerial surface protein ActA, primarily involved in host cell actin polymerization and intracellular bacterial motility (43, 68), contains at its N-terminal region a sequence (98-AIQVERRHPG-107) highly similar to a part of the pepPf1 peptide (Fig. 6). Mutation analysis of ActA demonstrated that the protein is involved in *L. monocytogenes* binding to, and subsequent uptake by, target cells through HSPG recognition functions.

Binding to heparin and HS is assumed to be dependent upon electrostatic interactions between positively charged regions of the ligand and the negatively charged sulfate groups of the GAG receptor (37). Like pepPf1, the ActA segment at positions 98 to 107 contains a positively charged motif, and this basic motif in pepPf1 has been shown to be necessary for HS binding (4, 63). In addition, the N-terminal region of ActA contains several other stretches with a high concentration of positively charged amino acids (mostly K and R) (Fig. 6), which form clusters of three or more basic residues very similar to those found in the heparin-binding domains of proteins which interact with sulfated GAG (34, 37, 65) (Fig. 6). Interestingly, the *Listeria ivanovii* ActA homolog LiactA, although only distantly related in terms of primary structure (29, 41), also has an N-terminal region which is rich in basic amino acids. Typically, HS-binding proteins contain several copies of the positively charged motifs (Fig. 6), a feature presumably favoring the interaction with the negatively charged HS receptor. Furthermore, secondary structure analyses predict that the clusters of basic amino acids at the N-terminal region of ActA are in α-helices. This is consistent with active domains in heparin-binding proteins usually being α-helical in nature, with the positively charged residues on one face of the helix thereby forming clusters of positive charges in the three-dimensional model (37). Helical wheel diagrams indicated that in some of the ActA α-helices which linearly contain dispersed cationic residues (e.g., ActA sequence 40-SRDIKELEKEKSNKV-52), positively charged amino acids form foci of high cationic density on one side of the helix (unpublished observations). Thus, ActA has a high copy number of motifs that could mediate HS recognition, and some of them appear to be suitably oriented in space to provide localized multivalency of positive charges to bind HS with sufficient affinity.

Therefore, ActA appears as a multifunctional protein involved in two important virulence-associated processes. There are many examples of multifunction among surface proteins in bacterial pathogens. For instance, *Yersinia* YadA mediates binding to many collagen types and to laminin and fibronectin, and the *Bordetella pertussis* filamentous hemagglutinin expresses binding specificities for CR3 integrins on macrophages and for galactose-containing glycoconjugates and heparin (45, 58, 73). Unusually, however, ActA would have two clearly distinct, important functions in two quite different compartments of the host, i.e., extracellularly by participating in listerial cytoadhesion and invasion and inside cells by directing actin assembly.

It has been shown recently that the domains required for actin polymerization and listerial actin-based motility reside within the N-terminal region of ActA (22, 44, 53). This region contains motifs related, albeit distantly, to sequences present in actin-binding proteins (10, 40). A segment essential for F-actin deposition has been identified between amino acids 99 and 122 of the mature protein. Within this region, there is a polycationic sequence, 117-KKRRK-121 (Fig. 6), which, according to Pistor et al. (53), could be involved in actin nucleation. As discussed above (Fig. 6), similar charged motifs are also present in heparin-binding domains of HS-binding proteins (37, 65). For instance, within the primary structure of epidermal growth factor-like growth factor, there is a heparin-binding motif which contains the sequence 31-KRKKK-35 (34), very similar to that present in ActA (Fig. 6). In addition, the highly charged ActA sequence 54-LKEKAEK-60 (Fig. 6) is similar to the actin-binding sites of caldesmon (LKEKQ) (72), villin (LKKEK) (23), and β-thymosin (LKKTET) (69). Therefore, some clusters of positively charged residues which in ActA could be involved in actin accumulation may also function as heparin-binding domains. The possible overlap between the putative actin-binding and HS-binding domains of ActA could have consequences for regulation of actin polymerization and listerial intracellular motility.

The observations reported in this paper emphasize that, as in other bacterial pathogens, the mechanisms by which *L. monocytogenes* locates, binds, and invades host tissues and cells are complex. The internalin system has been shown to play an important role in *L. monocytogenes* entry into normally nonphagocytic cells and in cell tropism (11, 24, 26). Lectin-like ligands mediating binding to D-glucosamine, L-fucosylamine, or *p*-aminophenyl-α-D-mannopyranoside residues have also been evidenced in *L. monocytogenes* (7). In this scenario, recognition of the HSPG receptor would be one among many events in the process of listerial interaction with the plasma membrane of target cells. Electrostatic interactions between HS and positively charged residues in ActA could lead to low-stringency binding to structurally heterogeneous populations of cell surface proteoglycan receptors which are widely distributed in mammalian body tissues. This low-affinity binding could be the basis of the wide host range of *L. monocytogenes*. It could also favor adequate presentation of other listerial ligands to their high-affinity signaling receptors, possibly responsible for cell tropism and for triggering cytoskeletal rearrangements and phagocytosis. This two-step model, in which HSPG act as preliminary receptors or as coreceptors, has been proposed for fibroblast growth factor interaction with target plasma membranes (39, 59). However, we have also shown here that fucoidan, keratan sulfate, and chondroitin sulfate, in spite of being negatively charged sulfated glycoconjugates, were not able to inhibit listerial attachment and invasion (Fig. 1), indicating that binding to HS is more than a simple electrostatic interaction between charged molecules of different polarities. Since sulfated glycoconjugates are both species and tissue specific, it is possible that listerial ligands responsible for interaction with HSPG recognize with increased affinity a particular class of HS structures.

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