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The Hek Outer Membrane Protein of *Escherichia coli* Strain RS218 Binds to Proteoglycan and Utilizes a Single Extracellular Loop for Adherence, Invasion, and Autoaggregation[▽]

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***Escherichia coli* is the principal gram-negative causative agent of sepsis and meningitis in neonates. The pathogenesis of meningitis due to *E. coli* K1 involves mucosal colonization, transcytosis of epithelial cells, survival in the bloodstream, and eventually invasion of the meninges. The last two aspects have been well characterized at a molecular level. Less is known about the early stages of pathogenesis, i.e., adhesion to and invasion of epithelial cells. We have previously reported that the Hek protein causes autoaggregation and can mediate adherence to and invasion of epithelial cells. Here, we report that Hek-mediated adherence is dependent on binding to glycosoaminoglycan, in particular, heparin. The ability to hemagglutinate, autoaggregate, adhere, and invade is contingent on a putative 25-amino-acid loop that is exposed to the outside of the bacterial cells.**

Escherichia coli is the principal gram-negative causative agent of sepsis and meningitis in neonates and is second only to the group B streptococci overall. The strains that cause these devastating infections are collectively known as neonatal meningitic *E. coli* (NMEC), and among NMEC strains, those expressing the K1 capsular antigen are predominant (13). *E. coli* K1 accounts for up to 40% of *E. coli* sepsis and 80% of *E. coli* meningitic isolates (29, 48) and generally results in a much poorer clinical outcome than other NMEC strains (36). The development of meningitis by *E. coli* K1 is a complex and multifactorial process. Initial colonization of the mucosae is followed by invasion and transcytosis of epithelial surfaces and eventual accession to intravascular space (50). The bacteria can then survive in the bloodstream, resulting in bacteremia, which is a prerequisite for subsequent invasion of the blood-brain barrier (BBB) (25). Inflammation accompanies the invasion of the central nervous system and results in neuronal damage, loss of integrity of the BBB, and pleocytosis (25).

Invasion of brain microvascular endothelial cells (BMECs), which comprise the BBB, is a complex process, though over the last decade much progress has been made in establishing the bacterial and host factors that are involved (24, 25). BMECs are efficiently invaded by NMEC, and this process is dependent on the bacterial OmpA outer membrane protein binding to a homolog of gp96 (43, 45, 46). In addition to its role as an adhesin, OmpA also protects NMEC from immune defenses, since these bacteria can multiply in macrophages and survive in

serum (42, 52, 54, 55). The CNF1 toxin also promotes bacterial uptake (22). For both CNF1 and OmpA, bacterial uptake is mediated by the induction of host cell signaling pathways (21). A series of outer membrane proteins known as the Ibes and other proteins, such as TraJ, AslA, and YijP, contribute to invasion of the meninges (16–19). Aside from outer membrane proteins, fimbriae (type 1 fimbriae and S fimbriae) also have roles in attachment to BMECs (29, 44, 59). The K1 capsule is required for survival during traversal of the BBB (15, 23) and has a role in establishing high-level bacteremia (26).

Apart from endothelial cells, NMEC is known to interact with epithelial cells. NMEC has been shown to invade bladder epithelial cells, and this process requires cytoskeletal rearrangements (37). However, it is thought that the gastrointestinal tract is the more essential site for initial colonization leading to subsequent invasive disease (50). Transcytosis of gastrointestinal cells, such as T84 or Caco-2, by NMEC has been demonstrated and was maximal when cells were differentiated and polarized (3). In common with bladder cell invasion, transcytosis of gastrointestinal cells was also dependent on cytoskeletal rearrangements. The factors that contribute to gastrointestinal colonization, or more particularly invasion, have not been unequivocally identified. However, a number of novel factors, the so-called Dgcs, have been shown to be required for colonization (35).

We have previously shown that the Hek protein is expressed by NMEC strain RS218 and is localized to the outer membrane (9). This protein can cause agglutination of red blood cells and can mediate autoaggregation. In addition, *E. coli* bacteria expressing the protein can adhere to and invade epithelial cells. However, it is unknown what the protein binds to or what amino acid sequences within the protein are required for adhesion/invasion.

Glycosoaminoglycans (GAGs) are large linear molecules composed of disaccharide repeats of hexosamine and sulfated

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TABLE 1. *E. coli* plasmids used in this work

Plasmid	Relevant details	Reference
pBSKII	Ap^r; ColE1 replicon	Stratagene
pHEK6	Ap^r; 866 bp of RS218 DNA carrying the *hek* gene cloned into pBSK II	9
pLoop1	Ap^r; derivative of pHEK6 with the DNA sequence corresponding to the first loop of the Hek protein replaced with CGCGCG	This work
pLoop2	Ap^r; derivative of pHEK6 with the DNA sequence corresponding to the second loop of the Hek protein replaced with GCGCGC	This work
pLoop3	Ap^r; derivative of pHEK6 with the DNA sequence corresponding to the third loop of the Hek protein replaced with GCGCGC	This work
pLoop4	Ap^r; derivative of pHEK6 with the DNA sequence corresponding to the fourth loop of the Hek protein replaced with GCGCGC	This work
pTia5	Ap^r; *tia* gene from pET125 subcloned into pBSKII	9
pTm23	Ap^r; derivative of pHEK6 with the DNA sequence corresponding to transmembrane strand 3 deleted.	This work

galactose or hexuronic acid (2). The GAG chains are usually covalently linked to a "core" transmembrane protein, thus forming a proteoglycan. GAGs can be grouped into different classes (heparin, heparan sulfate, dermatan sulfate, and chondroitin sulfate) based on the sugar composition and degree of modification (2). Proteoglycans are expressed by all nucleated cells and are often targets for binding by bacteria (49). Several pathogens, including *Neisseria gonorrhoeae* (56), *Bordetella pertussis* (38), *Mycobacteria* spp. (38), and *Listeria monocytogenes* (1), express surface proteins that recognize GAGs. Enterotoxigenic *E. coli*, a causative agent of traveler's diarrhea, also expresses a GAG-binding protein termed Tia (10, 11). Hek and Tia are 62% identical, though much of the similarity between the proteins is in the transmembrane regions (12). To our knowledge, Tia is not encoded by strains that have the *hek* gene.

Here, we show that the receptor for Hek is proteoglycan, in particular, heparinated proteoglycan. The ability to hemagglutinate, autoagglutinate, adhere, and invade is contingent on a putative 25-amino-acid loop that is exposed to the outside of bacterial cells. To our knowledge, this is the first factor from NMEC that utilizes this mechanism for adherence and invasion.

(Portions of this work were presented at the 155th and 152nd meetings of the Society for General Microbiology.)

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *E. coli* strain XL-1 Blue was used throughout this study. Bacterial plasmids are listed in Table 1. Bacteria were routinely cultured in Luria (L) broth or on L agar plates (39), and stocks were maintained in L broth supplemented with 8.7% (vol/vol) dimethyl sulfoxide at −70°C. Strains were also grown in Dulbecco's modified Eagle's medium (DMEM) or on Columbia agar plates containing 5% defibrinated horse blood when indicated in the text. Where appropriate, antibiotics were added to media at the following concentrations: carbenicillin, 50 μg/ml, and tetracycline, 30 μg/ml.

Eukaryotic cell lines and growth conditions. All cell lines used were obtained from ATCC (Manassas, VA). The mammalian cells lines used were CHO-K1 (ATCC CCL-61) and pgsA-745 (ATCC CRL-2242) cells. Stocks of cell lines were maintained in cell freezing medium-dimethyl sulfoxide (Sigma-Aldrich) under liquid nitrogen. All cell lines were grown in a 1:1 mixture of DMEM and Ham's F-12 medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Life Technologies) at 37°C in 5% CO$_2$.

Recombinant DNA techniques. Plasmid DNA was isolated using the Genelute Plasmid Miniprep kit (Sigma-Aldrich) or the Qiagen Plasmid Midi kit, and total genomic DNA was routinely purified using the Puregene genomic DNA purification kit (Gentra Systems) according to the manufacturers' instructions. Re-striction endonucleases were purchased from New England Biolabs and used according to the manufacturer's instructions. Standard methods were used for the ligation of DNA fragments and transformation of plasmid DNA (5, 7). The synthesis of oligonucleotides and all DNA sequencing were performed by MWG Biotech. Deletions within the *hek* gene of pHEK6 were made by inverse PCR with Phusion polymerase using the primers listed in Table 2. These primers contain the sequence CGC at their 5′ ends; when the products of inverse PCR were religated, this resulted in a new BssHII site (CGCGCG). This sequence encodes arginine followed by alanine, which is a turn-promoting sequence. The products of inverse PCR were ligated and transformed into *E. coli* XL-1 Blue. The deletions were verified by DNA sequencing.

Preparation and analysis of membrane proteins. Samples enriched for outer membrane proteins were prepared as previously described (4). Briefly, 12 optical density at 600 nm (OD$_{600}$) units (corresponding to 4 ml of a culture with an OD$_{600}$ of 3) of bacteria were harvested by centrifugation at 6,000 × *g* for 10 min and resuspended in 600 μl of sonication buffer (10% sucrose, 50 mM Tris-Cl [pH 7.5], 100 mM NaCl, 1 mM EDTA) and lysed by sonication. Intact bacteria were removed by centrifugation at 9,300 × *g* for 5 min, and the supernatants were incubated with 0.5% sarcosyl for 30 min with continuous mixing to solubilize the inner membranes. The sarcosyl-insoluble fraction containing the outer membranes was harvested by centrifugation at 21,000 × *g* for 30 min and resuspended in 100 μl of Laemmli buffer. All protein samples were stored at −20°C and boiled for 5 min prior to use. Protein samples were separated on discontinuous denaturing polyacrylamide gels by the method of Laemmli (30) and visualized following staining with Coomassie brillliant blue R-250. Alternatively, proteins were transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore) using a Biometra Fastblot semidry transfer apparatus at 5 mA/cm^2 for 20 min. Protein transfer was confirmed by staining the membrane with Ponceau S. The membranes were dried and incubated with a polyclonal primary anti-Hek antiserum raised in rabbits against a purified maltose-binding protein–Hek fusion protein, diluted 1:2,000 in blocking buffer (3% nonfat powdered milk in phosphate-buffered saline [PBS] containing 0.01% Tween 20) for 1 h at room temperature, followed by a secondary horseradish peroxidase-linked anti-rabbit antibody diluted 1:20,000 for 30 min. The blot was developed using the SuperSignal West Pico chemiluminescent horseradish peroxidase substrate (Pierce).

TABLE 2. Oligonucleotide primers used in this study

Primer name	Sequence
loop1F	5′-GCG GTA TTC AGT GGC GGT ATT G-3′
loop1R	5′-GCG TGA CAT CAC AGA GGC ACC-3′
loop2F	5′-CGC GAG GTG TCA GTC AAC ACA CTA ATG-3′
loop2R	5′-CGC CTC CAG TTC TGT ACG AAC CGG-3′
loop3F	5′-CGC AAC TTC GCA TGG AGC CTT GG-3′
loop3R	5′-CGC GGC GTA GCC AAT CCC TGC-3′
loop4F	5′-CGC AAA AGT CAT GAC ATC ATG CTT GG-3′
loop4R	5′-CGC ATC AAG ATA GCG ATA GCT GAG-3′
Tm3F	5′-TTT TAC GCT CGT GGA AAA GCT GAT TCG-3′
Tm3R	5′-AAT ACC GCC ACT GAA TAC CGT ATC-3′

Amino-terminal sequencing of Hek. A sarcosyl-insoluble fraction containing the Hek protein was separated on a discontinuous denaturing gel and transferred to a Immobilon-P polyvinylidene difluoride membrane. The transferred protein was stained by Ponceau S, and the section of membrane containing Hek was excised. Sequencing was carried out by Alta Biosciences, Birmingham, United Kingdom.

Flow cytometry. Surface exposure of the Hek protein on bacterial cells was confirmed by flow cytometry using an anti-Hek polyclonal antiserum and a fluorescently labeled secondary antibody. Briefly, approximately 5×10^7 bacteria from an overnight culture were harvested at $18,000 \times g$ for 10 min, washed three times with PBS, and resuspended in PBS containing 2% (vol/vol) formaldehyde. Excess formaldehyde was removed by three successive washes with PBS, and the bacteria were then incubated overnight with a 1:10 dilution of absorbed anti-Hek antiserum at 4°C. The labeled bacteria were then washed three times with PBS and incubated with a 1:100 dilution of secondary fluorescein isothiocyanate-conjugated anti-rabbit antibody for 1 h at room temperature. The labeled samples were then washed again as before, and fluorescence was analyzed on a Beckman Coulter Epics XL flow cytometer according to the manufacturer's instructions.

Hemagglutination assays. The abilities of bacterial strains to agglutinate erythrocytes were determined using a 1% suspension of human blood containing 100 mM mannose. Overnight bacterial cultures were harvested by centrifugation at $15,800 \times g$ for 1 min and resuspended in PBS to an OD_{600} of 1.0. The bacteria were then serially twofold diluted with PBS in a final volume of 100 μl in a 96-well microtiter plate. To each well was added an equal volume of the 1% blood suspension, and the plate was incubated at room temperature for 2 h or at 4°C overnight to allow unagglutinated erythrocytes to settle out of suspension.

Autoaggregation assays. Overnight cultures were harvested by centrifugation, resuspended in PBS, and normalized to an OD_{600} of approximately 4.0. Five milliliters of each culture was then transferred to a Kahn tube, and 50-μl samples were taken from the surfaces of the cultures at regular intervals to determine the OD_{600}. Assays were performed in duplicate, and the rate of autoaggregation was determined by the mean decrease in OD over time. Rates of autoaggregation were determined using Kaleidagraph software.

Quantitative cell association and invasion assays. Cell association and invasion assays were performed as previously described (6). CHO-K1 and pgsA-745 cells were seeded into 12-well trays at densities of 3.0×10^4 and 6.0×10^4 cells per well, respectively, in DMEM/Ham's F-12 medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum at 37°C in 5% CO_2 and grown to confluence.

Bacterial strains were grown overnight, washed with PBS, and diluted 1:500 in warm tissue culture medium. All bacterial strains used in this study displayed similar growth profiles and reached similar ODs (as determined by spectrophotometry) following overnight growth. The exact inoculum sizes following dilution in tissue culture medium were determined by spreading dilutions on Columbia blood agar plates and were comparable for all bacterial strains used. Mammalian cell monolayers were washed once with warm PBS, and medium containing bacteria (1 ml) was added to each well. The infected cells were centrifuged at $600 \times g$ for 5 min to initiate contact between the bacterial and mammalian cells and incubated at 37°C in 5% CO_2 for 1 h to allow adhesion to and invasion of the cultured cells. Infected monolayers were washed twice with warm PBS to remove any nonadherent bacteria. To determine the total number of cell-associated bacteria, the monolayer was disrupted by treatment with 0.1% Triton X-100 and the released bacteria were enumerated by spreading dilutions on Columbia blood agar plates. To determine the number of intracellular bacteria, a standard gentamicin protection assay was performed (20). Following the 1-h infection, cells were incubated with medium containing gentamicin (100 μg/ml) for 90 min at 37°C in 5% CO_2, washed with PBS, and disrupted with 0.1% Triton X-100, and the released bacteria were enumerated as before. The cell association or invasion efficiencies were expressed as the percentage of bacterial inocula recovered from triplicate wells. Experiments were performed at least three times.

In experiments in which exogenous GAGs were tested as potential inhibitors of invasion, the bacterial inocula were prepared as described previously in media containing the inhibitor to be tested and incubated for 30 min to 1 h at 37°C prior to infection of the cultured monolayers. The experiments were then carried out as previously described.

Enzymatic treatment of cells. To further establish the significance of GAGs in the Hek-mediated invasion of epithelial cells, eukaryotic cells were treated with heparinase prior to gentamicin protection assays. CHO-K1 cells were incubated at 37°C with heparinase III from *Flavobacterium heparinum* (75 mU/ml) for 3 h prior to the addition of bacteria. Heparinase III is a heparin-degrading lyase that recognizes heparin sulfate proteoglycan as its primary substrate, specifically hydrolyzing the glycosidic linkage present in heparan sulfate (33). This lyase has been shown to fully remove heparin moieties from proteoglycan core proteins (11).

RESULTS

Hek directs the invasion of epithelial cells via interactions with GAGs. Many pathogenic organisms have been shown to utilize interactions with the ubiquitous mammalian cell surface proteoglycans to both colonize and invade host tissues (12, 14, 49, 56). As stated above, the Tia invasin has identity with the Hek protein, particularly in the eight transmembrane regions. Tia mediates invasion by attaching to mammalian cell surface proteoglycans (10). Interactions with proteoglycans generally involve the GAG chains, for example, heparin and chondroitin sulfate, which are linked to the core protein of the proteoglycan. To examine if such interactions were important for Hek-promoted invasion of epithelial cells, the ability of Hek-expressing bacteria to invade epithelial cells deficient in GAG production or with reduced GAG expression was examined. In the first instance, CHO-K1 cells were treated with heparinase and the ability of Hek-expressing bacteria to invade these cells was ascertained. Cells that had been treated with heparinase were less well invaded (Fig. 1A). In fact, there was a greater than 60% decrease in the frequency of invasion of CHO-K1 cells that had been treated with heparinase. pgsA-745 cells are a CHO-K1 derivative that produces underglycosylated proteoglycans (8). Hek- or Tia-expressing *E. coli* K-12 bacteria are efficiently internalized by CHO-K1 cells, as described previously (9); however, invasion by these bacteria was greatly reduced in the xylotransferase mutant cell line pgsA-745 (Fig. 1B). The magnitude of invasion for Hek-expressing bacteria was reduced 23-fold in pgsA-745 cells.

In order to identify the specific GAG moiety involved in Hek-mediated interactions with epithelial cells, the abilities of different soluble GAG molecules to inhibit Hek-promoted invasion of CHO-K1 cells were investigated. Inhibition was tested with heparan sulfate, heparin (a more highly sulfated form), and chondroitin sulfate, the most common GAGs found on mammalian epithelial cell surfaces. The synthetic carbohydrate dextran sulfate (which is not a GAG) was included as a negative control to discount nonspecific charge-charge interactions, as it has a mass/charge ratio similar to that of heparin, the most highly sulfated of the GAGs tested (41). When cells were incubated with 10 μg/ml of GAG, only heparin was found to inhibit uptake of Hek-expressing *E. coli* K-12 at a statistically significant level (Fig. 2A). Heparin reduced the number of intracellular bacteria by 7.8-fold, whereas heparan sulfate, chondroitin sulfate, and dextran sulfate reduced uptake by only 1.6-, 1.4-, and 1.6-fold, respectively. Inhibition of Hek-promoted invasion by heparin was dose dependent and highly sensitive, with considerable inhibition (2.5-fold) apparent with as little as 100 ng/ml heparin (Fig. 2B). This suggests that Hek interacts specifically with heparin GAG moieties. Interestingly, heparan sulfate efficiently inhibited invasion at 100 μg/ml but had little or no effect at 10 μg/ml (not shown), suggesting that Hek is more specific for the more highly sulfated forms of heparin.

Topology of the Hek protein. Amino-terminal sequencing of the Hek protein, isolated from a sarcosyl-insoluble outer membrane fraction, yielded the sequence KEGK, corresponding to
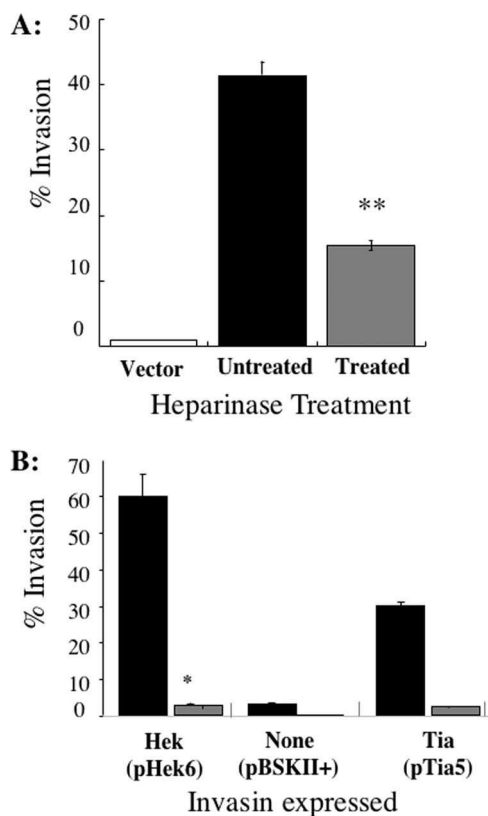
FIG. 1. Characterization of the Hek GAG receptor. (A) Invasion of CHO-K1 cells by *E. coli* K-12 carrying pBSKII (white bar) or pHEK6 (Hek+). Cells were treated with heparinase (gray bar) or not treated with enzyme (black bar). ∗∗, *P* < 0.005 versus untreated CHO-K1 cells. (B) Removal of heparin reduces Hek-mediated invasion. Invasion of CHO-K1 (black bars) or pgsA-745 (gray bars) cells by *E. coli* K-12 carrying pHEK6 (Hek+), pBSKII (vector control), or pTia5 (Tia+). ∗, *P* < 0.05 for invasion of pgsa-745 versus invasion of CHO-K1 cells by Hek-expressing bacteria. The error bars indicate standard deviations.
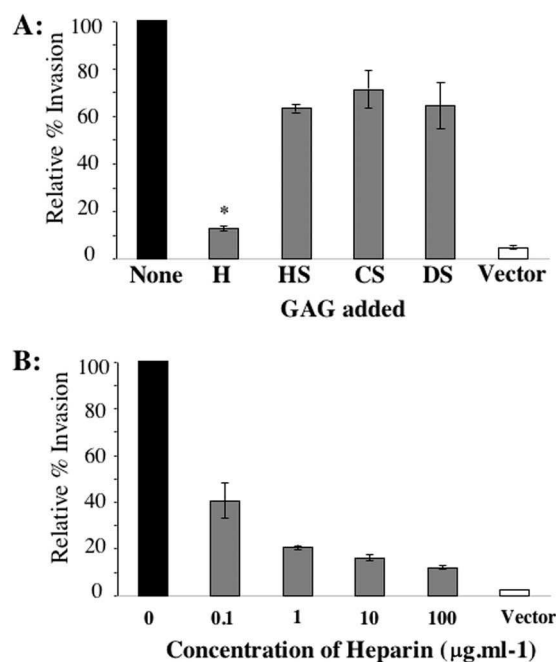

FIG. 2. Inhibition of Hek invasion by the addition of GAGs. (A) Inhibition of CHO-K1 invasion by *E. coli* K-12 harboring pHEK6 using exogenous GAGs. Shown is the invasion of CHO-K1 cells by *E. coli* K-12 carrying pHEK6 (black bar); *E. coli* K-12 carrying pHEK6 following incubation with 10 μg/ml of heparin (H), heparan sulfate (HS), chondroitin sulfate (CS), or dextran sulfate (DS) (gray bars); and *E. coli* K-12 harboring the vector control pBSKII. Bacteria were incubated for 30 min with each inhibitor prior to the assay. The non-GAG carbohydrate dextran sulfate was included as a negative control of nonspecific charge-charge interactions. Invasion efficiencies were expressed relative to the uninhibited control (normalized to 100%). ∗, *P* < 0.05 versus uninhibited control. (B) Inhibition of Hek-promoted invasion using exogenous heparin. Shown is invasion of CHO-K1 cells by *E. coli* K-12 carrying pHEK6 (black bar), *E. coli* K-12 carrying pHEK6 in the presence of various concentrations of heparin (gray bars), and *E. coli* K-12 harboring the vector control pBSKII (white bar). The error bars indicate standard deviations.

residues 23 to 26 of the predicted Hek sequence and consistent with the cleavage of 22 amino acids upon inner membrane translocation. The secondary structure of the 224-amino-acid mature Hek protein was modeled using several computational structural modeling tools, including PredictProtein (http://www.embl-heidelberg.de/predictprotein/predictprotein.html) and PORES (available upon request). The protein was predicted to contain eight β-strands separated by alternating short (3 to 6) or long (28 to 33) stretches of residues. A series of alternating hydrophobic amino acids occupying every second position along the strand can be found in each of the predicted β-strands of the Hek protein in a manner characteristic of β-barrel proteins (28). In support of the β-barrel prediction, several of the β-strands are also flanked by aromatic residues in a manner also characteristic of other β-barrel proteins (47, 51). Based on these observations, the Hek protein is likely to adopt a β-barrel conformation in the outer membrane consisting of eight antiparallel β-strands connected by four long external loops and three short periplasmic turns. The predicted external loops were also found to contain a very high number of charged residues compared to similar β-barrel proteins (27,

47, 58). Furthermore, the loops, or portions thereof, are predicted to be unstructured and disordered, as judged by using GlobPlot (32). Amino acids E31 to D48 (loop 1), Y80 to R93 (loop 2), W142 to N162 (loop 3), and S194 to K198 (loop 4) are predicted to be disordered, using Russell and Linding propensities for disorder. A topological model of the Hek protein is shown in Fig. 3, with the salient features highlighted, namely, the transmembrane β-strands and the surface-exposed charged residues.

The second surface-exposed loop of Hek is essential for all Hek-promoted phenotypes. In silico modeling of the Hek protein tertiary structure predicted the presence of four long external loops linking the eight strands of the protein barrel on the outer surface of the outer membrane. These four loops comprise the only predicted surface-exposed portion of the protein, and it can therefore be assumed that the biological function of the protein resides in one or more of these loops. In order to elucidate which of the loops play roles in the various Hek-promoted phenotypes, a series of *hek* loop deletion mutant genes were constructed, based on the pHEK6 plasmid, each lacking the coding sequence for a single loop. In
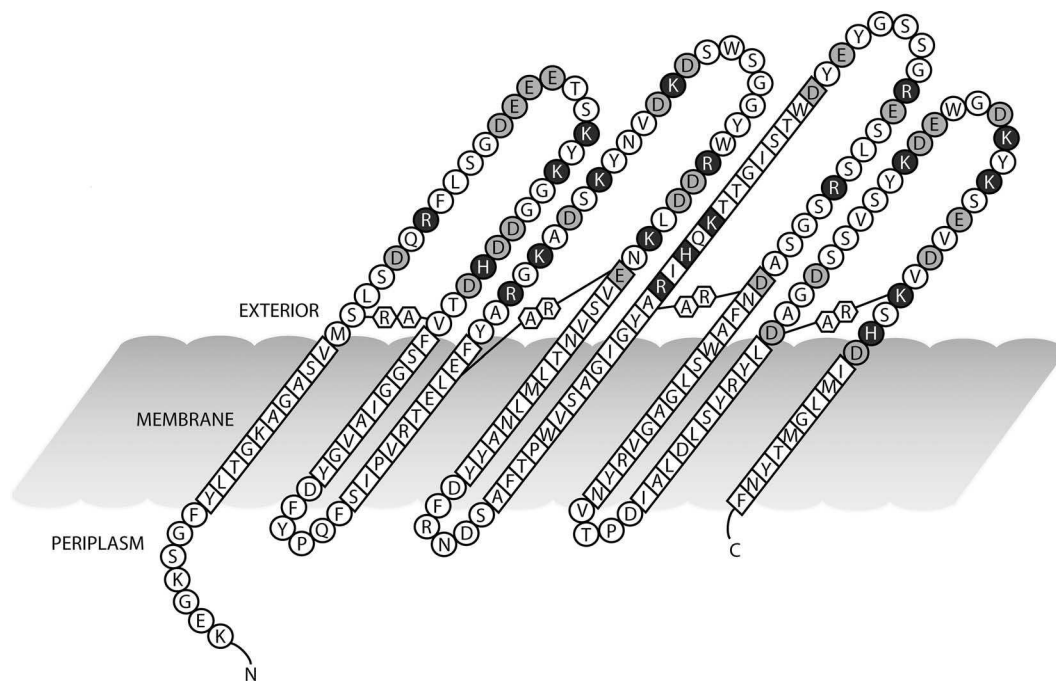
FIG. 3. Two-dimensional model of the topology of the membrane-spanning mature Hek protein. Residues predicted to belong to transmembrane β-strands are indicated with diamonds, and residues in unstructured loops are circled. The extent of each loop deletion mutation and the position of the substituted alanine-arginine (AR) bridge used in each are indicated by hexagonal residues. Positively charged residues located in the surface-exposed loops are highlighted in black, and negatively charged residues are in gray.

each case, the coding sequence for each loop was replaced with two codons encoding arginine (CGC) and alanine (GCG), and the extents of the resulting deletions are shown in Fig. 3. Expression and surface exposure of each mutant protein was confirmed by Western immunoblot analysis and flow cytometry on intact bacterial cells using a polyclonal Hek-specific antibody (Fig. 4A). All four mutant proteins were expressed. For those proteins lacking loop 1, 2, or 4, the level of expression and surface localization was similar to that of wild-type Hek. The mutant lacking the third loop was less well expressed than the others and could be detected on the surfaces of recombinant bacteria at a level 10-fold lower than that of wild-type Hek. The ability of each to promote hemagglutination, autoaggregation, and invasion of CHO-K1 cells was assessed (Fig. 4B and Table 3). Deletion of the first loop had no discernible effect on hemagglutination, autoaggregation, or invasion. Deletion of the third loop abolished measurable hemagglutination and autoaggregation. The decreased levels of adhesion and hemagglutination displayed by Hek lacking loop 3 can be directly attributed to the decreased copy number of the protein in the cell membrane. Notwithstanding this decrease in the copy number of the loop 3 deletion mutant, E. coli K-12 expressing the mutant protein still efficiently invaded CHO-K1 cells at a level more than 50% of wild type. This may indicate that there is a saturable level of Hek required for invasion. Deletion of the fourth loop resulted in a protein that gave intermediate levels of hemagglutination and autoaggregation. Interestingly, deletion of this loop resulted in a protein that conferred enhanced invasiveness on the bacteria. Thus, loop 4 may have a modulatory effect on invasion. Hek lacking loop 2 could not promote heamagglutination and had a much reduced

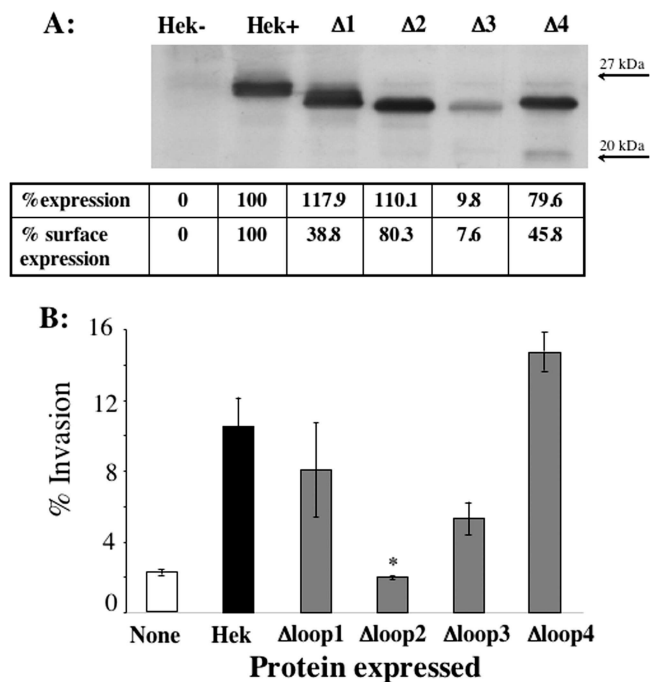| A:	Hek−	Hek+	Δ1	Δ2	Δ3	Δ4
% expression	0	100	117.9	110.1	9.8	79.6
% surface expression	0	100	38.8	80.3	7.6	45.8

FIG. 4. Identification of the functional loop of Hek. (A) Expression of the Hek loop deletion mutants (pLoop1 to -4) was confirmed by Western immunoblot analysis using a Hek-specific polyclonal antiserum. Densitometry values, normalized to the level of full-length Hek expression from pHEK6, are displayed below. The surface exposure of each was determined by flow cytometry on intact bacterial cells using the same anti-Hek antiserum and normalized to the fluorescence signal of bacteria expressing wild-type Hek. (B) Invasion of CHO-K1 cells by E. coli K-12 carrying pBSKII (white bar), pHEK6 (black bar), or each of the Hek loop deletion mutants (gray bars). The error bars indicate standard deviations.

TABLE 3. Hemagglutination and autoaggregation by
Hek loop mutants

Parameter	Hek⁻	Hek⁺	With loop deleted			
			1	2	3	4
Hemagglutination titer	0	16	16	0	0	8
Autoaggregation rate (10^{-3} OD_{600} units · min^{-1})	2.8	28.0	29.2	5.5	5.6	17.6

level of autoagglutination. The levels of invasion promoted by
the protein were at vector control levels. Thus, loop 2 appears
to be the most important region for all activities of the Hek
protein.

Deletion of putative transmembrane β-sheets 2 and 3. The
Tia protein can bind to heparin using the sequence AVGYD
FYQHYNVPVRTEVE from amino acids 51 to 69. In our
model of Hek, the corresponding sequence is predicted to be
the second and third membrane-spanning β-strands of Hek
(Fig. 5). A deletion derivative of pHEK6, pTm23, lacking the
DNA encoding the sequence AVGYDFYPQFSIPVRTELE,
from amino acids 51 to 69, was constructed. Although a trun-
cated derivative, HekΔ23, was expressed from this construct, it
did not insert into the outer membrane as judged by sodium
dodecyl sulfate-polyacrylamide gel electrophoresis of sarcosyl-
enriched fractions or by flow cytometry (data not shown).

DISCUSSION

The pathogenesis of *E. coli* K1 neonatal meningitis is a
complex and multifaceted process. A great deal is now known
about the mechanism by which these organisms disseminate in
the bloodstream, establishing septicemia, and breach the BBB.
Considerably less is known about the initial stages of infection,
in particular, colonization and invasion of the epithelia. We
have previously described the first *E. coli* K1 factor to be
implicated in both adhesion to and invasion of cultured human
gastrointestinal epithelial cells (9). Hek is a 26-kDa protein
that is predicted to form an eight-stranded β-barrel in the
outer membrane of *E. coli* K1 strain RS218. Many pathogens
have been shown to exploit host cell proteoglycans, in partic-
ular heparin-containing proteoglycans, in order to enable col-
onization and invasion of host tissues. Several proteins from
pathogenic bacterial species implicated in this process bear a
striking resemblance to Hek, including the Tia protein of en-
terotoxigenic *E. coli* and the broader family of Opa proteins of
Neisseria spp. (10, 57). Although these similarities are mostly
confined to amino acids predicted to be located in the mem-
brane-spanning β-barrel, we undertook to determine if Hek
could also utilize these ubiquitous host cell structures to pro-
mote adhesion to and invasion of cultured epithelial cells.

We have shown that expression of Hek in *E. coli* K-12 strains
is sufficient to promote invasion of the human gastrointestinal
epithelial cell line T84 and the model epithelial cell line
CHO-K1 (9). Our results suggest that this process requires
interactions with heparin-containing proteoglycans on the host
cell surface. Pretreatment of CHO-K1 cells with heparinase
significantly decreased the efficiency of Hek-dependent inter-
nalization. In keeping with this observation, *E. coli* K-12 ex-
pressing Hek or Tia was significantly less invasive in pgsA-745

cells, a derivative of CHO-K1 cells that produces underglyco-
sylated proteoglycans. Inhibition of invasion with a number of
soluble GAGs was also greatest with heparin, and this inhibi-
tion was dose dependent, suggesting a specific interaction with
highly sulfated heparin proteoglycans. Heparin was also the
only GAG found to inhibit the interaction of *E. coli* strain K1
with CHO-K1 cells (R. P. Fagan and S. G. J. Smith, unpub-
lished observations). However, *E. coli* K-12 expressing Hek or
Tia was still significantly more invasive than the negative con-
trol in pgsA-745 cells. This suggests that Hek and Tia may be
capable of interacting with additional CHO-K1 cell surface
components. It is possible that the initial interaction between
the bacterial protein and a GAG moiety facilitates further
interactions with an additional cell surface component, per-
haps even with the core protein of the receptor proteoglycan,
which would still be present on the surfaces of pgsA-745 cells.

Computational modeling of the Hek protein is consistent
with an eight-stranded β-barrel with three short periplasmic
turns and four longer surface-exposed loops. As these loops
are the only predicted surface-exposed regions of the proteins,
it is reasonable to assume that some or all of the loops are
involved in mediating the various Hek-dependent phenotypes
described to date (9). Although no secondary structure was
predicted in the surface-exposed loops, the β-strands on either
side of the third loop were predicted to extend a considerable
distance beyond the membrane surface. Such an arrangement
may serve to extend and support the loop farther from the
membrane and to provide a support structure for the other
loops in a manner similar to that observed with OpcA in
Neisseria meningitidis and OmpX in *E. coli* (47, 51, 58). One
striking feature of the surface-exposed loops of Hek is the
extreme abundance of charged amino acids present, 20 basic
and 26 acidic residues. The presence of surface-exposed basic
residues has been shown to be involved in binding to proteo-
glycans (31), and in the case of OpcA in particular, the com-
bination of extended β-strand structure and an abundance of
basic residues resulted in the formation of a positively charged
cleft that is theorized to be the site of binding to heparinated
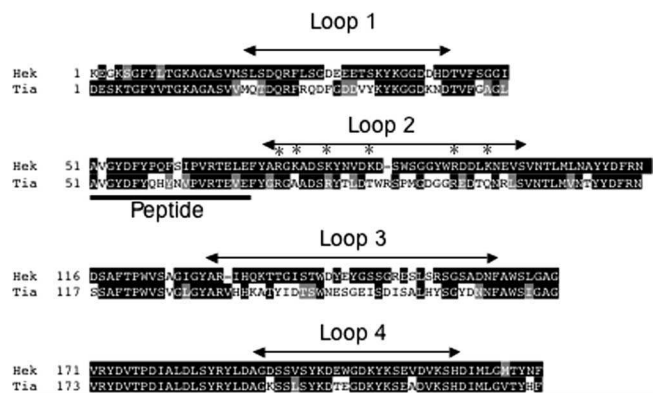proteoglycans (47). Although the data gathered here do not

FIG. 5. Comparison of the Hek and Tia proteins. The amino acid
sequences of the mature forms of Hek and Tia were aligned by ClustalW.
The positions of putative loops are indicated, and positively charged
residues within loop 2 are marked by asterisks. The position of the
peptide used to inhibit Tia adhesion (34) and binding of heparin (10)
is underlined.

allow any accurate prediction of the final structure of the surface-exposed loops of Hek, they may play a similar role in proteoglycan binding. Construction of a series of mutant Hek proteins, each lacking a single loop, has allowed a detailed analysis of the contribution of each to Hek-promoted hemagglutination, autoaggregation, and epithelial cell invasion. Deletion of a single 28-amino-acid loop, loop 2, was found to abrogate all three phenotypes. Individual deletions of the remaining three loops had little or no effect in the same assays. These data suggest that the second surface-exposed loop is essential for the interaction of Hek with its receptors, with only minor contributions from the remaining surface-exposed portions of the protein.

It has also been reported in the literature that the second loop of the related Tia protein is involved in invasion (34) and binding of heparin (10). The data are based on the abilities of two short peptides (GYDFYQHYNVPVRTEVEFY and AVGYDFYQHYNVPVRTEVEC) to inhibit invasion of HCT8 human epithelial cells by Tia-expressing bacteria. In addition, these peptides bind directly to heparin-albumin-biotin. The region corresponding to these peptides in Hek is predicted to be a transmembrane β-strand and is rich in hydrophobic residues. In support of this prediction, a deletion of this region in Hek yields a protein that is incapable of folding correctly and inserting into the outer membrane. If this sequence in Hek is also to engage in contacts with GAGs, it would suggest that the β-barrel may have a pore of sufficient size to permit access of the GAG chains. There is currently no evidence to support this hypothesis.

Hek is not restricted to NMEC isolates. In one study, 55% of urinary tract isolates were shown to have the *hek* gene (53). Given that the Hek homolog Hra1 can bind to cells under shear stress, it is tempting to speculate if Hek in these isolates can promote uroepithelial adherence. Furthermore, we have identified a protein in the recently completed sequence of the enteroaggregative *E. coli* strain 042 that is 90% identical to Hek. While aggregative adherence in enteroaggregative *E. coli* is mediated by fimbriae (40), considering the aggregative nature of Hek-mediated adherence, it may be that Hek contributes to this phenomenon. In addition, we have recently identified *hek* in 36% of a cohort of 221 bacteremic isolates of *E. coli*.

Hek can contribute to invasion of epithelial cells, and we have recently established that the OmpA and IbeB proteins of *E. coli* K1 also mediate entry into gastrointestinal epithelial cells (R. P. Fagan and S. G. Smith, unpublished data). For *E. coli* causing meningitis, invasion may thus be a multifactorial process involving several outer membrane adhesin/invasin proteins.

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