67-kDa Laminin Receptor Promotes Internalization of Cytotoxic Necrotizing Factor 1-expressing *Escherichia coli* K1 into Human Brain Microvascular Endothelial Cells

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*Escherichia coli* K1 is the most common Gram-negative organism causing meningitis, and its invasion of human brain microvascular endothelial cells (HBMEC) is a prerequisite for penetration into the central nervous system. We have reported previously that cytotoxic necrotizing factor 1 (CNF1) contributes to *E. coli* K1 invasion of HBMEC and interacts with 37-kDa laminin receptor precursor (37LRP) of HBMEC, which is a precursor of 67-kDa laminin receptor (67LR). In the present study, we examined the role of 67LR in the CNF1-expressing *E. coli* K1 invasion of HBMEC. Immunofluorescence microscopy and ligand overlay assays showed that 67LR is present on the HBMEC membrane and interacts with CNF1 protein as well as the CDPGYIGSR laminin peptide. 67LR was up-regulated and clustered at the sites of *E. coli* K1 on HBMEC in a CNF1-dependent manner. Pretreatment of CNF1+ *E. coli* K1 with recombinant 37-kDa laminin receptor precursor reduced the invasion rate to the level of ∆cnf1 mutant, and the invasion rate of CNF1+ *E. coli* K1 was enhanced in 67LR-overexpressing HBMEC, indicating 67LR is involved in the CNF1+/E. coli K1 invasion of HBMEC. Coimmunoprecipitation analysis showed that, upon incubation with CNF1+ *E. coli* K1 but not with ∆cnf1 mutant, focal adhesion kinase and paxillin were recruited and associated with 67LR. When immobilized onto polystyrene beads, CNF1 was sufficient to induce internalization of coupled beads into HBMEC through interaction with 67LR. Taken together, this is the first demonstration that *E. coli* K1 invasion of HBMEC occurs through the ligand-receptor (CNF1–67LR) interaction, and 67LR promotes CNF1-expressing *E. coli* K1 internalization of HBMEC.

The successful entry of invasive bacterial pathogens into the nonphagocytic cells such as endothelial and epithelial cells is the key to the establishment of several infectious diseases, including bacterial meningitis. *Escherichia coli* K1 is the major cause of neonatal bacterial meningitis, a serious illness associated with considerable mortality and morbidity (1, 2). Most cases of bacterial meningitis develop as the result of hematogenous spread, but the mechanism(s) by which circulating bacteria cross the blood-brain barrier (BBB) is not completely understood.

*E. coli* K1 invasion of human brain microvascular endothelial cells (HBMEC), constituting the BBB, is a prerequisite for its penetration into the central nervous system in vivo (3, 4). Previous studies showed that *E. coli* K1 traversal of the BBB requires a high degree of bacteremia, binding to and internalization into HBMEC accompanying actin cytoskeleton rearrangements and related signaling pathways and traversal of the BBB as live bacteria (4). Several *E. coli* structures are necessary for *E. coli* K1 invasion of HBMEC (e.g. the K1 capsule, CNF1, and Ibe proteins, AsIA and TraJ), but it is unclear how all of these *E. coli* determinants contribute to HBMEC invasion *in vitro* and crossing of the BBB in vivo.

Cytotoxic necrotizing factor 1 (CNF1) is a bacterial virulence factor associated with pathogenic *E. coli* strains causing meningitis and urinary tract infection (5). CNF1 is an AB-type toxin, composed of the N-terminal receptor-binding domain and the C-terminal catalytic domain possessing a deamidase activity. Through the site-specific deamidation of a Glu residue to Gln, CNF1 has been shown to permanently activate small GTP-binding proteins of the Rho family such as RhoA, Rac, and Cdc42. CNF1 has been suggested to be internalized via receptor-mediated endocytosis upon binding to a cell surface receptor by a clathrin-independent and caveolin-independent mechanism (6). Once endocytosed, CNF1-containing vesicles are delivered to the late endosome in a microtubule-dependent manner, and its C-terminal catalytic domain is translocated into the cytosol by the acidic pH-dependent manner to activate RhoGTPases (6). CNF1 induces uptake of latex beads, bacteria, and apoptotic body into nonprofessional phagocytes such as epithelial and endothelial cells by macropinocytosis (7). Recent studies demonstrate that CNF1 exploits ubiquitin-proteasome machinery to restrict RhoGTPase activation, resulting in uropathogenic *E. coli* infection of bladder cells (8). We have reported previously (9) that CNF1 is a virulence factor contributing to *E. coli* K1 invasion of HBMEC in *vitro* and traversal of the BBB in vivo. Treatment of HBMEC with CNF1 enhances *E. coli* K1 invasion in the RhoA-dependent pathway (9). However, it is unclear how CNF1-expressing *E. coli* invades HBMEC.

Recently, a 37-kDa laminin receptor precursor (37LRP) has been identified as a CNF1-interacting protein from screening

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1 The abbreviations used are: BBB, blood-brain barrier; HBMEC, human brain microvascular endothelial cells; CNF1, cytotoxic necrotizing factor 1; 37LRP, 37-kDa laminin receptor precursor; 67LR, 67-kDa laminin receptor; FAK, focal adhesion kinase; PBS, phosphate-buffered saline; GST, glutathione S-transferase; PVDF, polyvinylidene difluoride; r37LRP, recombinant 37LRP; PI3K, phosphatidylinositol 3-kinase.
the cDNA library of HBMEC by the yeast two-hybrid system using the N-terminal domain of CNF1 as bait. In addition, the level of 37LRP expression is correlated with the cellular activities of CNF1 such as RhoA activation and bacterial uptake in HBMEC (10). 37LRP, also known as p40, has been reported to be a ribosome-associated cytoplasmic protein and precursor of the 67-kDa laminin receptor (67LR). Even though the structure and maturation mechanism by which the 67LR is synthesized from the precursor is unclear, mature 67LR is present on the cell surface and functions as a membrane receptor for the adhesive basement membrane protein laminin (11). 67LR has been found in a variety of cell types including brain endothelial cells. 67LR has been reported as the receptor for cellular prion protein and certain alphaviruses including Sindbis and Venezuelan equine encephalitis viruses for their binding and entry into mammalian host cells (12–14). Its increased expression is known as a prognostic marker for the invasive and metastatic tumors because of its enhanced expression in a wide variety of tumors including breast, ovary, lung, colon and prostate carcinomas, and lymphomas (11).

In this study, we examined the role of 67LR in the internalization of CNF1-expressing E. coli K1, and we demonstrated that the 67LR is an HBMEC protein interacting with CNF1, and their interaction promotes internalization of CNF1-expressing E. coli K1 and CNF1-immobilized beads into HBMEC.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Reagents, and Antibodies—**CNF1+ E. coli K1 E44 strain is a spontaneous rifampin-resistant mutant derived from the cerebrospinal fluid isolate of a neonate with meningitis, strain RS218 (serotype O18:K1:H7, CNF1 cerebrospinal fluid isolate of a neonate with meningitis, strain RS218. The isogenic Δcnf1 deletion mutant was generated and reported previously (9). Anti-CNFI monoclonal antibodies (N6G1 and JC4) were obtained from Dr. A. O’Brien (Unified Services University of the Health Sciences, Bethesda). The laminin-based nonapeptide Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg (CDPGYIGSR) and recognizing antibody were obtained from Dr. H. K. Kleinman (National Institutes of Health). Monoclonal (MLuC5) and polyclonal (Ab711) antibodies recognizing 67LR were purchased from NeoMarkers (Union City, CA) and Abcam Limited (Cambridge, UK), respectively. Anti-actin antibody was obtained from Sigma. Antibodies against FAK and paxillin were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY) and Immunocytometry Systems, respectively.

**HBMEC—**Primary HBMEC were isolated and cultured as described previously (15). Cells were grown on collagen-coated flasks in RPMI medium containing 10% heat-inactivated fetal bovine serum, 10% NuSerum, 2 mM glutamine, 1 mM pyruvate, penicillin (100 units/ml), streptomycin (100 μg/ml), essential amino acids, and vitamins. Transfection of HBMEC with 37LRP was performed as described previously (10). Briefly, a p6DAS1.1 vector harboring full-length 37lrp was delivered into HBMEC using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen), and transfectants were cloned after selection with G418 sulfate (800 μg/ml).

**Purification of Reconstituted CNF1—**Purification of reconstituent GST-CNFI was performed as described previously with a modification (10). Briefly, full-length cfnf1 was cloned into pGEX-2T (Amersham Biosciences) and introduced into E. coli XLI-Blue. Expression of GST-CNFI was induced by incubation of culture in the presence of isopropyl 1-thio-β-D-galactopyranoside for 5 h at 25 °C. Bacteria were harvested by centrifugation, resuspended in PBS with protease inhibitor mixture, and lysed by sonication. After another centrifugation, the bacterial lysate was incubated in 8 μl urea for 30 min with rotation. The supernatant after centrifugation was resolved by SDS-PAGE to examine the expression of CNF1. The supernatant containing GST-CNFI was dialyzed in serial dilutions of urea for refolding. GST-CNFI in the final 2 μl urea was incubated with glutathione-coupled agarose beads overnight at 4 °C with rotation and poured into a column. The column was washed with PBS, and bound GST-CNFI was eluted with glutathione according to the manufacturer’s protocol (Clontech). The presence of GST-CNFI was confirmed by Western blot analysis with anti-CNFI monoclonal antibody, and its functional activity was verified with the rhotein assay described previously (10).

**Ligand Overlay Assay—**Confluent monolayers of HBMEC were washed with ice-cold PBS and lysed at 4 °C in RIPA buffer as described previously (8). Briefly, whole cell lysates were separated in SDS-polyacrylamide gel and transferred to a PVDF membrane. After washing, the membrane was blocked with TBST (25 mM Tris, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) containing 4% skimmed milk for 1 h at 4 °C and incubated overnight with CNF1 or CDPGYIGSR laminin peptide at 4 °C. After extensive washing, bound CNF1 and laminin peptide were detected with anti-CNFI (NG8) and anti-CDPGYIGSR peptide antibodies, respectively. The membranes were subsequently visualized using an enhanced chemiluminescence kit (Amersham Biosciences).

**Immunofluorescence Microscopy—**HBMEC were grown on the collagen-coated glass coverslips until they reached confluence. Cells were incubated with either the parent E. coli E44 strain or isogenic Δcnf1 mutant for 30 min at 37 °C, and unbound bacteria were removed by extensive washing with PBS. Subsequently, cells were fixed and processed for immunofluorescence staining as described previously (16). 67LR and E. coli K1 were stained with MLuC5 and anti-OmpA antibodies, respectively. The binding of primary antibodies was visualized by incubation with Alexa-488 and Texas Red-conjugated secondary antibodies (Molecular Probes, Eugene, OR). After washing with PBS, cells were mounted and examined with the fluorescence microscope (Olympus), and images were processed with Adobe Photoshop.

**Determination of 67LR on the HBMEC Surface—**HBMEC grown in collagen-coated 24-well plates were incubated with CNF1+ E. coli K1 E44 strain or Δcnf1 mutant at 37 °C. After 30 min of incubation, HBMEC were washed three times with PBS and fixed with 4% paraformaldehyde for 15 min. Cells were washed twice with PBS and incubated with anti-67LR (MLuC5) antibody for 1 h, followed by incubation with Alexa-488 conjugated secondary antibody for 1 h. Antibodies were diluted in PBS containing 10% normal goat serum. The cells were washed and immediately analyzed with a fluorescence spectrometer equipped with plate reader (Molecular Devices, Sunnydale, CA). Each set was run in triplicate.

**Bacterial Invasion and Association Assays—**Bacterial invasion assays with gentamicin treatment were performed as described previously (16). Briefly, confluent cultures of HBMEC grown in 24-well plates were incubated with 105 of either the parent CNF1+ E. coli K1 E44 strain or isogenic Δcnf1 mutant in experimental medium at 37 °C for 1 h. After incubation, HBMEC were washed three times with PBS and fixed with 4% paraformaldehyde for 15 min. Cells were washed twice with PBS and incubated with anti-67LR (MLuC5) antibody for 1 h, followed by incubation with Alexa-488 conjugated secondary antibody for 1 h. Antibodies were diluted in PBS containing 10% normal goat serum. The cells were washed and immediately analyzed with a fluorescence spectrometer equipped with plate reader (Molecular Devices, Sunnydale, CA). Each set was run in triplicate.

**Commmunoprecipitation—**HBMEC were lysed in RIPA buffer at 4 °C as described above. Samples were centrifuged at 10,000 x g for 20 min at 4 °C, and supernatants were collected for protein quantification. An equal amount of protein (500 μg) was incubated with protein A-beads for 1 h at 4 °C to remove nonspecifically bound proteins. After centrifugation, the supernatant was incubated with anti-67LR (Ab711) antibody for 2 h at 4 °C and further incubated with protein A-beads for another 1 h at 4 °C. Protein A-beads were washed with RIPA without deoxycholate, resolved by SDS-PAGE, and transferred onto a PVDF membrane. The blots were blocked with TBST containing 4% skimmed milk. After blocking, the membranes were incubated with primary antibody and horseradish peroxidase-linked secondary antibody and subsequently visualized using an enhanced chemiluminescence kit (Amersham Biosciences).

**Binding and Internalization Assays with CNF1-immobilized Polystyrene Beads—**Carboxylated fluorescent beads (1 μm in diameter, Molecular Probes, Eugene, OR) were covalently coupled with GST-CNFI (100 μg of protein) according to the manufacturer’s protocol (Molecular Probes). GST alone was used to couple to beads as a negative control. HBMEC grown in collagen-coated 24-well plates were incubated with either CNFI- or GST-coupled beads (107/ml) for 1 h at 4 or 37 °C for binding or association assays, respectively. After washing three times with ice-cold PBS to remove unbound beads, the fluorescence intensity of bound beads in each well was measured with a fluorescence spectrometer equipped with plate reader (Molecular Devices). To determine the internalization of beads, HBMEC were incubated with coupled beads for 1 h at 37 °C. After washing three
**RESULTS**

The cnf1 Deletion Mutant (Δcnf1) of E. coli K1 Is Defective in Invasion but Not in Binding to HBMEC—CNF1 has been shown to contribute to E. coli K1 invasion of HBMEC in vitro and traversal of the blood-brain barrier in the experimental hematogenous meningitis animal model (9). To determine whether decreased invasion of the Δcnf1 mutant is because of their defective binding to the target cells, cell association and invasion assays were carried out with CNF1+ E. coli K144 strain and Δcnf1 mutant (Fig. 1). The invasion frequency of the Δcnf1 mutant was reduced by 60% compared with that of E44, which is consistent with our previous report (9). However, the binding ability of the Δcnf1 mutant was comparable with that of E44, indicating that CNF1 participates in the E. coli K1 internalization of HBMEC and is not involved in bacterial binding to HBMEC.

CNF1 Interacts with 67LR of HBMEC—We have shown previously that the 37LRP of HBMEC interacts with CNF1 of E. coli K1, and its expression level affects the activities of CNF1 such as RhoA activation and bacterial uptake (10). 37LRP is shown to be a cytoplasmic protein and matured to 67LR, which is known to be present on the cell surface and binds to laminin with high affinity (17). Therefore, we examined whether CNF1 interacts with the 67LR of HBMEC. 67LR of HBMEC was detected with anti-67LR (Ab711) antibody (Fig. 2A, left). Ligand overlay assay showed that CNF1 interacted with a polypeptide showing the same molecular weight with 67LR (Fig. 2A, middle). Because a laminin-based CDPGYIGSR peptide has been shown to interact specifically with 67LR, we used this peptide to detect 67LR (18). When applied, the laminin CDPGYIGSR peptide also bound to the polypeptide, whose molecular weight is the same as that interacting with anti-67LR antibody and CNF1 (Fig. 2A, right). These findings indicate that CNF1 of E. coli K1 interacts with 67LR of HBMEC.

Next, we examined the localization of 67LR in HBMEC using anti-67LR (MLuC5) monoclonal antibody. Immunofluorescence microscopy demonstrated that 67LR was abundant and present in the cytoplasm as well as cell membrane of the subconfluent culture of HBMEC (Fig. 2B). In contrast, the confluent culture of HBMEC monolayer exhibited much less total 67LR expression, most of which was found in the basolateral side of monolayer where cells are interacting with extracellular matrix (Fig. 3C). These results indicate that 67LR expression depends on the confluence of the HBMEC monolayer, which is consistent with the report published previously (19) showing that 67LR expression is inhibited by contact inhibition. Taken together, our results indicate that CNF1 from E. coli K1 is able to interact with 67LR of the HBMEC monolayer.

CNF1+E. coli K1 Induces 67LR Clustering around the Bacteria on the Cell Surface of HBMEC—We have reported previously (9, 10) that CNF1 is a major bacterial determinant contributing to E. coli K1 invasion of HBMEC. To examine whether 67LR of HBMEC is involved in CNF1-expressing E. coli K1 invasion, confluent HBMEC monolayers were incubated with CNF1+E. coli K1E44 strain for 15 min at 37 °C, fixed, permeabilized, and stained with anti-67LR (MLuC5) to detect 67LR. The apical side of the confluent HBMEC monolayer showed very weak 67LR staining, and most 67LR was found at the basolateral side of the monolayer (Fig. 3C). However, 67LR was evident on the apical surface of the HBMEC monolayer where CNF1+E. coli K1 was located,
exhibiting 67LR clusters on HBMEC (Fig. 3B). In order to assess whether 67LR clusters are colocalized with adherent E. coli K1, the HBMEC monolayers incubated with CNF1-expressing E. coli K1 (E44) strain for 15 min were fixed, permeabilized, and immunostained with anti-67LR (MLuC5) monoclonal antibody (A–C). Phase contrast (A) and corresponding fluorescence (B) images showed that 67LR was clustered at the sites of E. coli K1 on the apical side of the HBMEC monolayer (arrow). The basal side of HBMEC monolayer showed more expression of 67LR (C). Clustering of 67LR was examined in unpermeabilized HBMEC incubated with CNF1+/E44 (D–F), HBMEC were incubated with anti-OmpA and anti-67LR antibodies to detect E. coli K1 and 67LR, respectively. Primary antibodies were visualized with secondary antibodies conjugated with Alexa-688 (bacteria, D) or Alexa-488 (67LR, E). Superimposed image (F) showed that 67LR was clustered at the bacterial binding sites on the apical side of unpermeabilized HBMEC monolayer. Bar indicates 10 μm.

Next, we examined whether the formation of 67LR clustering requires CNF1 expression in E. coli K1 (Fig. 4). In contrast to CNF1+ E. coli K1 E44 strain, the isogenic Δcnf1 mutant did not induce 67LR clustering on the apical side of the unpermeabilized HBMEC monolayer even though it bound to HBMEC with comparable efficiency (Fig. 4, D–F). These findings indicate that CNF1+/E44 (A–C) was exhibited on the apical side of the HBMEC monolayer, whereas Δcnf1 mutant bound on the apical side of HBMEC did not induce 67LR clustering. Bar indicates 5 μm.

FIG. 3. 67LR is clustered at E. coli K1-binding sites on HBMEC. HBMEC grown on the collagen-coated coverslips were incubated with CNF1-expressing E. coli K1 (E44) for 15 min. HBMEC were fixed, permeabilized, and immunostained with anti-67LR (MLuC5) monoclonal antibody (A–C). Phase contrast (A) and corresponding fluorescence (B) images showed that 67LR was clustered at the sites of E. coli K1 on the apical side of the HBMEC monolayer (arrow). The basal side of HBMEC monolayer showed more expression of 67LR (C). Clustering of 67LR was examined in unpermeabilized HBMEC incubated with CNF1+/E44 (D–F), HBMEC were incubated with anti-OmpA and anti-67LR antibodies to detect E. coli K1 and 67LR, respectively. Primary antibodies were visualized with secondary antibodies conjugated with Alexa-688 (bacteria, D) or Alexa-488 (67LR, E). Superimposed image (F) showed that 67LR was clustered at the bacterial binding sites on the apical side of unpermeabilized HBMEC monolayer. Bar indicates 10 μm.

FIG. 4. 67LR clustering on the surface of HBMEC is dependent on the CNF1 of E. coli K1. HBMEC incubated with CNF1+/E. coli K1 (E44) or isogenic Δcnf1 mutant for 15 min were fixed and immunostained without permeabilization. E. coli K1 (A and D) and 67LR (B and E) were visualized with anti-OmpA and anti-67LR (MLuC5) antibodies, respectively. The superimposed images (C and F) demonstrated that 67LR clustering at the binding site of CNF1+/E44 (A–C) was exhibited on the apical side of the HBMEC monolayer, whereas Δcnf1 mutant bound on the apical side of HBMEC did not induce 67LR clustering. Bar indicates 5 μm.
67LR is up-regulated in HBMEC incubated with CNF1-expressing E. coli K1 but not with Δcnf1 mutant. HBMEC were incubated with either CNF1+ E. coli K1 E44 strain or Δcnf1 mutant for the indicated times at 37 °C. A, the whole cell lysates (30 μg each) were resolved with SDS-PAGE and transferred onto the membrane. The total amount of 67LR was detected with anti-67LR (Ab711) antibody. Total expression of 67LR was increased continuously by CNF1+ E44 but not by Δcnf1 mutant of E. coli K1, indicating that 67LR is up-regulated in a CNF1-dependent manner. Actin was detected as loading control. B, HBMEC incubated with bacteria were washed and fixed with 4% paraformaldehyde. The fixed cells were incubated with anti-67LR (MLuC5) antibody for 1 h, followed by incubation with Alexa-488-conjugated secondary antibody for 1 h. The fluorescence intensity was measured with a fluorescence spectrometer as described under “Experimental Procedures.” The 67LR expressed on the apical side was increased by 2-fold in HBMEC incubated with CNF1+ E44 but not by Δcnf1 mutant of E. coli K1.

HBMEC monolayer was determined as described under “Experimental Procedures.” As shown Fig. 5B, the amount of 67LR expressed on the apical side was increased by 2-fold in HBMEC incubated with CNF1+ E44, compared with nonincubated HBMEC. The up-regulation of 67LR was evident after 30 min of incubation and was maintained up to 60 min. However, the 67LR expression remained unchanged in HBMEC incubated with theΔcnf1 mutant, indicating that CNF1 of E. coli K1 is responsible for the 67LR up-regulation, especially on the apical surface of HBMEC monolayer.

67LR Is Required for the CNF1-dependent E. coli K1 Invasion of HBMEC—Our previous report (10) demonstrated that CNF1-mediated RhoA activation and bacterial uptake are modulated by exogenous r37LRP or transfection of HBMEC with 37LRP. Therefore, we investigated whether CNF1 interaction with 67LR is required for HBMEC invasion by CNF1+ E. coli K1. In vitro bacterial invasion assays with gentamicin treatment were performed in the presence of r37LRP to block the interaction between CNF1 and 67LR. Pretreatment of the CNF1+ E. coli K1 E44 strain with r37LRP decreased the invasion rate by 50% compared with GST control pretreatment. In contrast, the invasion rate of the Δcnf1 mutant was not affected by r37LRP (Fig. 6A). These results indicate that exogenous r37LRP competes against 67LR of HBMEC and reduces CNF1+ E. coli K1 invasion to the level of the Δcnf1 mutant. Next, invasion assays were carried out with 37LRP-transfected HBMEC, which have demonstrated previously (10) greater CNF1 binding than the control pcDNA vector-transfected HBMEC. The 37LRP-transfected HBMEC exhibited greater 67LR expression and enhanced invasion rate of the E44 strain by 2-fold, compared with the pcDNA transfected cells (Fig. 6B and inset). In contrast, invasion rates of the CNF1-negative laboratory strain HB101 remained low and unchanged in 37LRP-transfected HBMEC. These findings indicate that the level of 67LR expression affects CNF1+ E. coli K1 invasion of HBMEC. Taken together, our results illustrate that 67LR is involved in CNF1+ E. coli K1 invasion of HBMEC.

E. coli K1 Enhances 67LR Association with FAk and Paxillin in a CNF1-dependent Manner—E. coli K1 invasion has been shown previously (21, 22) to induce tyrosine phosphorylations of FAk at tyrosine 397 and two downstream effectors, paxillin and PI3K, all of which are involved in actin cytoskeleton rearrangements involved in bacterial entry into HBMEC. FAk is a nonreceptor kinase but, upon phosphorylation by E. coli K1, has been shown to be accumulated at the invading E. coli K1 (21). Because 67LR was clustered at the sites of CNF1+ E. coli K1, we examined whether 67LR is able to recruit and associate with FAk in HBMEC incubated with CNF1+ E. coli K1. Confluent HBMEC monolayers were incubated with either CNF1+ E. coli K1 E44 strain or Δcnf1 mutant for the indicated times, and communoprecipitation was performed. Total 67LR of whole cell lysates were precipitated with anti-67LR (Ab711) antibody, and the amount of FAk associated with 67LR was determined as described under “Experimental Procedures.” As shown in Fig. 7A, FAk association with 67LR was increased at 15 min and reached a peak after 30 min of incubation with CNF1+ E. coli K1 E44 strain. However, HBMEC incubated with theΔcnf1 mutant did not show any increase in the amount of FAk associated with 67LR (Fig. 7A). These findings suggest that E. coli K1 induces FAk association with 67LR in a CNF1-dependent manner. We next examined paxillin, which has been shown to be a downstream effector of FAk phosphorylation and associated with FAk during E. coli K1 invasion of HBMEC. Paxillin was also recruited to the 67LR of HBMEC incubated with CNF1+ E. coli K1 E44 strain for 30 min but not with Δcnf1 mutant (Fig. 7B). Our results indicate that CNF1+ E. coli K1 induces recruitment of FAk and paxillin to the 67LR in a CNF1-dependent manner.
**CNF1 Is Sufficient to Induce Association and Internalization of Coupled Beads into HBMEC through Interaction with 67LR.**—Our findings demonstrated that 67LR is the cellular receptor for CNF1 of *E. coli* K1 and promotes CNF1-grown *E. coli* K1 invasion of HBMEC. To assess whether 67LR-CNFI interaction is sufficient to induce bacterial invasion, we utilized polystyrene beads (1 μm in diameter) covalently coupled with recombinant CNF1, and we examined the beads for their abilities to bind and internalize into HBMEC, as described under “Experimental Procedures.” When incubated with HBMEC for 1 h at 37°C, CNF1-coupled beads exhibited significantly greater association and internalization into HBMEC compared with control bovine serum albumin-coupled beads (Fig. 8A). Approximately half (58%) of the CNF1-coupled beads associated with HBMEC were found to be internalized (Fig. 8A). Their association and internalization were inhibited by preincubation of CNF1-coupled beads with anti-CNFI monoclonal antibodies (Fig. 8A). These findings indicate that CNF1 is sufficient to induce association and internalization of coupled beads into HBMEC. We next examined whether 67LR is involved in CNF1 beads binding to HBMEC. HBMEC were pretreated with anti-67LR (MLu5) antibody for 1 h at 4°C and further incubated for 1 h with CNF1 beads at 4°C. CNF1 beads were able to bind the confluent HBMEC monolayer at 4°C, and their binding was inhibited by pretreatment of HBMEC with anti-67LR antibody in a dose-dependent manner (Fig. 8B). In contrast, their binding was not affected by pretreatment with control antihemagglutinin antibody (Fig. 8B). These findings illustrate that CNF1 immobilized on the beads is sufficient for inducing association and internalization of coupled beads into HBMEC through their interaction with 67LR.

**Internalized CNF1-coupled Beads Are Delivered to Lysosomes.**—Previous studies (16) demonstrated that *E. coli* K1 modulates intracellular trafficking of bacteria-containing vacuoles to prevent fusion with lysosomes through the K1 capsule-dependent mechanism in HBMEC. To determine whether CNF1 affects intracellular trafficking and lysosomal fusion, the maturation of vacuoles containing CNF1 beads was investigated by dual staining immunofluorescence microscopy. HBMEC were incubated with CNF1-coupled beads for 90 min at 37°C and stained with antibodies recognizing Lamp-1 and cathepsin D, markers for late endosomes/lysosomes and lysosomes, respectively. Internalized CNF1-coupled beads were found in the membrane-bound vacuoles and acquired Lamp-1 and cathepsin D, indicating delivery of bead-containing vacuoles to lysosomes of HBMEC (Fig. 9, C and F). These findings suggest that CNF1 is unlikely to be involved in prevention of vacuolar fusion with lysosomes, which has been demonstrated with CNF1-expressing *E. coli* K1.
CNF1-expressing E. coli K1 triggers clustering and up-regulation of 67LR and induces association of 67LR with FAK and paxillin in a CNF1-dependent manner. We also show that CNF1, through interaction with 67LR, is sufficient to induce internalization of coupled beads into HBMEC without affecting intracellular trafficking.

Previous studies have shown that CNF1 pretreatment activates small GTP-binding proteins of the Rho family such as Rho, Rac, and Cdc42 by deamidation of a specific glutamine residue. As consequence of this activation, CNF1 induces the rearrangement of the actin cytoskeleton and promotes an intense and generalized membrane ruffling activity. This leads, in turn, to the induction of a phagocytic-like behavior (macropinocytosis) in nonprofessional phagocytes such as epithelial and endothelial cells, provoking uptake of large particles including bacteria, inert beads, and apoptotic bodies (7). These findings suggest that macropinocytosis may be the mechanism for CNF1-expressing E. coli entry into eukaryotic cells. However, our findings in this study clearly demonstrate that CNF1-expressing E. coli K1 internalization into HBMEC occurs via its interaction with 67LR, as shown by the following: (a) CNF1 protein interacted with 67LR; (b) 67LR present on the HBMEC surface was up-regulated and clustered at the sites of E. coli K1 in a CNF1-dependent manner; (c) the invasion rate of CNF1-expressing E. coli K1 was reduced by addition of r37LRP to the level of the Δcnf1 mutant, whereas invasion rate of the Δcnf1 mutant was not affected by r37LRP; and (d) CNF1-expressing E. coli K1 invasion was significantly increased in the 67LR-overexpressing HBMEC. Taken together, our results suggest that internalization of CNF1-expressing E. coli K1 is mediated by a ligand-receptor (CNF1–67LR) interaction rather than macropinocytosis, which is characterized as a receptor-independent endocytosis mechanism. This concept is supported by our previous report demonstrating that CNF1+ E. coli K1 invades HBMEC through a zipper-like mechanism as shown by transmission electron microscopy, and its invasion of HBMEC is decreased by monodansylcadaverine, an inhibitor of receptor-mediated endocytosis (24).

Time-dependent up-regulation of 67LR has been shown in human cell lines derived from breast cancer and melanoma when interacting with extracellular matrix protein such as laminin (20). Immunoelectron microscopy using anti-67LR antibody and colloidal gold particles demonstrated translocation of the cytoplasmic receptor to the cell membrane after treatment of melanoma cells with laminin (23). For example, laminin binding to cell surface 67LR induces a rapid, protein synthesis-dependent increase of 67LR, indicating that 67LR interaction

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**FIG. 8.** CNF1-coupled beads are able to associate with and internalize into HBMEC. A, CNF1-coupled fluorescent beads pretreated with anti-CNF1 monoclonal antibodies (JC4 and NG8) were incubated with HBMEC for 1 h at room temperature, and their association and internalization were determined as described under “Experimental Procedures.” The association and internalization of CNF1-coupled beads were inhibited by anti-CNF1 antibodies. *, p < 0.01, compared with total association of CNF1 beads alone; **, p < 0.01, compared with internalization of CNF1 beads alone. B, HBMEC were pretreated with either anti-67LR (MLuC5) or anti-hemagglutinin monoclonal antibody at 4 °C. After 1 h of incubation, HBMEC were further incubated with CNF1-coupled beads for 1 h at 4 °C. The binding of CNF1 beads was inhibited by anti-67LR antibody in a dose-dependent manner but not by anti-hemagglutinin antibody. *, p < 0.01, compared with CNF1 beads alone.

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**FIG. 9.** Internalized CNF1-coupled beads are delivered to lysosomes. HBMEC incubated with CNF1-coupled fluorescence beads for 90 min at 37 °C were fixed, permeabilized, and stained with anti-Lamp-1 (left) or anti-cathepsin D (right) antibodies. CNF1-coupled beads were detected by their own fluorescence (red). Superimposed images demonstrated that internalized CNF1-coupled beads were colocalized with Lamp-1 (C) and cathepsin D (F), markers for late endosomes and lysosomes (arrows), respectively, indicating that CNF1-coupled beads were delivered to the lysosomes of HBMEC. Bar indicates 10 μm.
with its ligand, laminin, enhances the level of 67LR expression in a positive feedback. In this study, we showed that CNF1+ E. coli K1 induced 67LR up-regulation in HBMEC. However, the Δcnf1 mutant failed to exhibit 67LR up-regulation even though both bacterial strains exhibited comparable binding abilities to HBMEC. These findings indicate that CNF1-expressing E. coli K1 contributes to 67LR up-regulation in HBMEC, but the underlying mechanisms are unclear. Most interestingly, increased expression of the 67LR has been shown to be important in invasive and metastatic properties of a wide variety of tumors, including breast, lung, ovary, colon, and prostate carcinomas and lymphoma (11). This is the first demonstration of identifying CNF1 as a bacterial ligand in 67LR up-regulation in HBMEC. Additional studies are needed to determine how CNF1-expressing E. coli K1 induces 67LR up-regulation in HBMEC and whether CNF1-expressing E. coli K1 has any role in malignant transformation of certain tumors.

E. coli K1 invasion of HBMEC requires rearrangements of the actin cytoskeleton, as shown by the F-actin condensation associated with invading E. coli K1 and blockade of bacterial invasion by cytochalasin D (24). We have reported previously that E. coli K1 triggers two signal transduction pathways involved in actin cytoskeleton rearrangements: (a) phosphorylation of FAK, its associated cytoskeletal protein paxillin, and P13K; (b) activation of RhoGTPases and cPLA2 (4). E. coli K1 determinants such as OmpA and FimH are suggested to be involved in the phosphorylations of FAK and its downstream effectors, paxillin and P13K (21). In contrast, CNF1 of E. coli K1 has been demonstrated to be responsible for activation of RhoA in HBMEC (9, 10). However, it is unknown how the aforementioned signaling molecules are recruited to the sites of bacterial entry and involved in E. coli K1 internalization into HBMEC. Our results showed for the first time that 67LR is induced to form clusters at the sites of adherent E. coli K1 and associated with FAK and paxillin in a CNF1-dependent manner, which is consistent with our previous findings showing the accumulation of FAK and F-actin at the site of E. coli K1 internalization (21, 24). Taken together, we speculate that 67LR, upon interaction with CNF1+ E. coli K1, forms a complex with FAK and paxillin at the bacterial entry sites on HBMEC and triggers actin cytoskeleton rearrangements, leading to bacterial internalization into HBMEC. This concept is supported by our recent findings that E. coli K1 activates phosphorylation of ezrin in a CNF1-dependent mechanism and recruits phosphorylated ezrin to bacterial entry sites, resulting in association of invading bacteria with microvilli-like membrane protrusions on the surface of HBMEC.² At present, it is unclear how 67LR interacts with FAK and paxillin and how 67LR clusters induce actin cytoskeleton rearrangements. However, there are several reports (26, 27) demonstrating that the 67LR is associated with the actin cytoskeleton. Massia et al. (28) have demonstrated that 67LR is clustered and colocalized with actin-binding proteins such as α-actinin and vinculin, when human foreskin fibroblasts are spreading on 67LR-binding laminin peptide (GYIGSRY). These investigators have proposed that 67LR is initially uncoupled from the cytoskeleton, especially actin microfilament bundles, but its binding to a ligand such as laminin invokes association of the receptor-ligand complex with the cytoskeleton via actin-binding proteins such as α-actinin and vinculin (28). These findings suggest that 67LR clustering induced by CNF1+ E. coli K1 also interacts with the actin microfilament bundle through association with actin-binding proteins such as FAK and paxillin. Most interestingly, CNF1 treatments of bovine aortic endothelial cells and Swiss 3T3 cells have been reported to activate tyrosine phosphorylation of FAK and paxillin through the Rho-dependent pathway (25, 29). Thus, it may be possible that phosphorylation of FAK occurs through the CNF1-mediated signal transduction pathway. Studies are in progress to elucidate the signal transduction pathways downstream of 67LR interaction with CNF1-expressing E. coli K1.

We demonstrated in this study that the CNF1–67LR interaction promotes the CNF1-expressing E. coli K1 internalization of HBMEC by the ligand-receptor (CNF1–67LR) interaction. However, it remains unclear how 67LR of HBMEC interacts with CNF1 of E. coli K1 because CNF1 is a bacterial cytoplasmic protein and is not secreted into the culture medium (5, 9). CNF1 does not contain any identifiable signal sequence (type 2 secretion mechanism) and does not belong to an operon, like the hly operon that encodes its own secretory apparatus (type 1 secretion mechanism) (5). Moreover, components of a putative type III secretion system have not been found in the genome of E. coli K1 strain R5218 (www.genome.wisc.edu). Nevertheless, the results with CNF1-immobilized beads clearly demonstrate that CNF1 is sufficient to induce internalization of coupled beads through specific interaction with 67LR on the HBMEC surface. Therefore, we propose that the initial association of E. coli K1 with HBMEC through other bacterial determinants such as type 1 fimbrae (FimH) and OmpA may induce translocation of CNF1 to the bacterial surface, which will subsequently interact with 67LR to mediate CNF1-expressing E. coli K1 invasion of HBMEC. This hypothesis is supported by our preliminary observation showing that CNF1 is detected with anti-CNF1 monoclonal antibody on the surface of E. coli K1 when associated with HBMEC but not on the surface of free bacteria.³ Additional studies are needed to elucidate how CNF1 is translocated to interact with 67LR present on the surface of HBMEC.

CNF1-coupled beads were found to be internalized and delivered to lysosomes in HBMEC, as determined by accumulation of cathepsin D in the bead-containing vacuoles. We have demonstrated previously that internalized CNF1-expressing E. coli K1 were found in the membrane-bound vacuoles and did not fuse with lysosomes, resulting in maintaining their viability inside vacuoles. In contrast, vacuoles containing the K1 capsule-deficient mutant (ΔneuDB, cnf1+) fused with lysosomes and the K1 capsule-deficient mutant exhibited lysosomal degradation and reduced invasion rate, indicating that the K1 capsule is responsible for modulation of intracellular vacuolar trafficking and enhancing intracellular bacterial survival in HBMEC (16). Taken together with the comparable binding ability of the Δcnf1 mutant, CNF1 is suggested to participate in the bacterial entry step rather than association or intracellular survival of E. coli K1 in HBMEC.

Our results showed that CNF1 plays an important role in E. coli K1 internalization into HBMEC. However, the invasion rate of the Δcnf1 mutant was decreased by 60% compared with that of the wild-type bacteria, suggesting that E. coli K1 invasion of HBMEC involves other bacterial determinants. We have shown previously that several other bacterial determinants (e.g., the K1 capsule, the Ibe proteins, TraJ and AslA) contribute to E. coli K1 invasion of HBMEC in vitro as well as translocation across the blood-brain barrier in vivo (3, 4). These determinants are suggested to be involved in the CNF1-independent bacterial invasion. Studies are currently in progress to examine how these determinants participate in E. coli K1 internalization into HBMEC.

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² K. J. Kim, D. Goti, C.-H. Teng, T. Dam, and K. S. Kim, submitted for publication.

³ K. J. Kim, unpublished data.
In conclusion, our results show that 67LR interacts with CNF1, and the interaction promotes CNF1-expressing E. coli K1 internalization into the HBMEC.

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