The morbidity and mortality associated with *Escherichia coli* K1 meningitis during the neonatal period have remained significant over the last decade and are once again on the rise. Transcytosis of brain microvascular endothelial cells (BMEC) by *E. coli* within an endosomoid to avoid lysosomal fusion is crucial for disemination into the central nervous system. Central to *E. coli* internalization of BMEC is the expression of OmpA (outer membrane protein A), which interacts with its receptor on the actin organization that leads to invasion. However, nothing is known about the nature of the signaling events for the formation of endosomes containing *E. coli* K1. We show here that *E. coli* K1 infection of human BMEC (HBMEC) results in activation of caveolin-1 for bacterial uptake via caveolae. The interaction of caveolin-1 with phosphorylated protein kinase Ca (PKCa) at the *E. coli* attachment site is critical for the invasion of HBMEC. Optical sectioning of confocal images of infected HBMEC indicates continuing association of caveolin-1 with *E. coli* during transcytosis. Overexpression of a dominant-negative form of caveolin-1 containing mutations in the scaffolding domain blocked the interaction of phospho-PKCa with caveolin-1 and the *E. coli* invasion of HBMEC, but not actin cytoskeleton rearrangement or the phosphorylation of PKCa. The interaction of caveolin-1 with phospho-PKCa was completely abrogated in HBMEC overexpressing dominant-negative forms of either focal adhesion kinase or PKCa.

Treatment of HBMEC with a cell-permeable peptide that represents the scaffolding domain, which was coupled to an antennapedia motif of a *Drosophila* transcription factor significantly blocked the interaction of caveolin-1 with phospho-PKCa and *E. coli* invasion. These results show that *E. coli* K1 internalizes HBMEC via caveolae and that the scaffolding domain of caveolin-1 plays a significant role in the formation of endosomes.

A broad range of pathogens have the capacity to induce their own uptake by host cells via classical endocytosis (1, 2). Because the endocytic pathway mediated by caveolae appears to avoid fusion of lysosomes, pathogens often utilize this pathway to survive within the host cells. Caveolae are indentations in the plasma membrane thought to be involved in transcytosis, signal transduction, and uptake of membrane components and extracellular ligands. Although it is known that caveolae have the capacity to pinch off as endocytic vesicles and can internalize a variety of ligands, the process seems to be selective under normal culture conditions. Caveolae contain a distinct group of molecules, including cholesterol, a 22-kDa protein, caveolin-1 and various glycolipids, and glycosylphosphatidylinositol-anchored molecules (3–8). Furthermore, receptors for various growth factors and hormones, including epidermal growth factor, platelet-derived growth factor, and insulin, have been localized in caveolae (9–12). Caveolin-1 is assumed to take a hairpin-loop conformation in the lipid bilayer, thereby exposing both the N and C termini to the cytoplasmic surface (13). A stretch of amino acids referred to as the “scaffolding domain” within caveolin-1 interacts with many signaling proteins (3–5, 13). Although the mechanism of caveola formation for transcytosis of small molecules has been well established, it is not clearly known how bacterial pathogens induce signaling events that lead to caveola formation during invasion of host cells.

One of the crucial events in *Escherichia coli* meningitis is the traversal of *E. coli* across the blood-brain barrier (BBB). During this process, *E. coli* cells seek refuge in a safe compartment in human brain microvascular endothelial cells (HBMEC), which form a lining of the BBB. The BBB exhibits selective permeability mainly for transport of macromolecules, liquids, and nutrients between the blood and the brain. However, *E. coli* could exploit the mechanisms of transport of macromolecules to promote their entry. Using HBMEC culture as an *in vitro* model of the BBB, we have demonstrated that *E. coli* invasion is a complex, multifactorial process that involves important virulence factors like S-fimbriae, OmpA, IbeA, and IbeB (14–18). However, our studies so far have suggested that OmpA interaction with endothelial cells via a gp96-like receptor on HBMEC is critical for invasion (19). OmpA-mediated *E. coli* invasion of HBMEC induces the phosphorylation of FAK and its interaction with phosphatidylinositol 3-kinase (20, 21).

We have further shown that the invading *E. coli* cells induce phosphorylation of PKCa in a phosphatidylinositol 3-kinase-dependent manner and that PK Ca is recruited to the plasma membrane, where it interacts with its substrate, MARCKS (myristoylated alanine-rich C-kinase substrate) (22). These signaling events lead to the accumulation of actin beneath the *E. coli* entry site, which is required for the generation of lamel-
lipodia (23). These protrusions of HBMEC enwrap the E. coli cell, which is progressively drawn into a compartment in the host cell. However, the nature of the compartment in which the E. coli cell resides in order to cross the cell is not known.

The interaction of phospho-PKCα with caveolin-1 has been shown to be crucial for the formation of caveolae, which contain a conserved consensus phosphorylation site for activated PKCα (24–26). Thus, the increased PKCα activity that we observed during E. coli invasion could be directed toward initiation of the interaction with caveolin-1, which may play a key role in regulating the internalization of E. coli. Previous studies have shown that pathogens like E. coli expressing FimH antigen and Chlamydia trachomatis prefer caveola-dependent endocytosis to invade eukaryotic cells and actively recruit caveolin to the sites of bacterial entry (1, 2, 27). In addition, the internalization of SV40 via caveolae has been shown to trigger actin rearrangements and dynamin recruitment to the sites of entry (28–32). These events appear to depend on the presence of cholesterol and on the activation of tyrosine kinases that phosphorylate proteins in caveolae. However, these studies used only general tyrosine kinase inhibitors and thus did not reveal the nature of specific kinases involved in caveola formation.

This report describes, for the first time, the role of PKCα in initiating caveolin-1-mediated uptake of E. coli into HBMEC via caveolae. During E. coli invasion, the activated PKCα migrated to the cell membrane and interacted with caveolin-1, which co-localized with condensed actin beneath the bacterial entry site. Interference of the PKCα interaction with caveolin-1, either by overexpression of a dominant-negative mutant form of caveolin-1 or by introduction of a peptide that represses the scaffolding domain into HBMEC, significantly reduced the invasion. In addition, we also demonstrate the association of caveolin-1 with internalized E. coli by confocal microscopy.

**EXPERIMENTAL PROCEDURES**

**Bacteria**—A rifampin-resistant mutant of E. coli K1 strain RS218 (serotype O18:K1:H7), E44, has been isolated from the cerebrospinal fluid of a neonate with meningitis and invades HBMEC in a cell culture model. E91 is a noninvasive derivative of E44 in which the invasion could be directed toward initiation of the PKCα activity, confluent monolayers of HBMEC grown on collagen-coated dishes (60-mm diameter) were washed with RPMI 1640 medium, and E. coli cells suspended in experimental medium were added. Following stimulation for varying periods of time (0, 5, 10, 15, and 30 min), the cells were rinsed twice in ice-cold phosphate-buffered saline, and placed on ice. The cells in each 60-mm dish were harvested by scraping on ice into 2 ml of cell homogenization buffer consisting of 20 mM Tris (pH 7.5), 0.25 mM sucrose, 10 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 2 mM diethiolethol. The cells were subjected to mild sonication, and the cell lysates in the above buffer were initially centrifuged at 5000 × g to remove the debris, followed by centrifugation at 100,000 × g at 4 °C for 45 min. The supernatant from this step was designated as the cytosolic fraction, and the pellet was designated as the membrane fraction. The procedures for immunoprecipitation, Western blotting, and scanning of the bands were described previously (22).

**Preparation of Cytosolic and Membrane Fractions of HBMEC**—For detection of PKCα activity, confluent monolayers of HBMEC grown on collagen-coated dishes (60-mm diameter) were washed with RPMI 1640 medium, and E. coli cells suspended in experimental medium were added. Following stimulation for varying periods of time (0, 5, 10, 15, and 30 min), the cells were rinsed twice in ice-cold phosphate-buffered saline, and placed on ice. The cells in each 60-mm dish were harvested by placing the tubes in a boiling water bath. After adding 80% glycerol 'C for 30 min. The reactions were stopped by placing the tubes in a boiling water bath. After adding 80% glycerol (1 μl), the samples were loaded onto an 0.8% agarose gel in 50 mM Tris–HCl (pH 8.0) and separated in the same buffer at 100 V for 15 min.

**Fluorescence Staining—HBMEC**—HBMEC were grown in eight-well collagen-coated dishes (60-mm diameter) were washed with RPMI 1640 medium containing gentamicin (100 μg/ml) for 1 h to kill extracellular bacteria. The monolayers were washed again and lysed with 0.5% Triton X-100. The intracellular bacteria were enumerated by plating on sheep blood-agar plates. In duplicate experiments, the total cells counted had been determined as described previously except that the gentamycin step was omitted. In some experiments, HBMEC were pretreated with various inhibitors for 30 min prior to the addition of bacteria. The effects of these inhibitors on HBMEC were assessed by the trypan blue exclusion method, and the effects on bacterial viability were tested by colony plate counting (10). The cell monolayers were fixed with 2% paraformaldehyde before assaying for fluorescence intensity.

**Immunofluorescence Staining—HBMEC**—HBMEC were grown in eight-well collagen-coated dishes, and infected and incubated at 37 °C in a humidified environment for 1 h at room temperature. They were fixed with phosphate-buffered saline and incubated with secondary antibodies conjugated to the fluorocein isothiocyanate, respectively, or rhodamine-phalloidin for 30 min at room temperature. The cells were washed again; the chambers were removed; and the
Inhibitors of Caveola Formation Significantly Reduce E. coli Invasion of HBMEC—Our previous studies showed that PKCα activated in E. coli infection is critical for actin accumulation at the site of E. coli entry (23). Thus, we speculated that PKCα might interact with caveolin-1, one of the molecules that has a PKC activation site and is a constituent of caveola. Thus, to examine the role of caveola, E. coli invasion assays were performed following treatment of HBMEC with various concentrations of filipin, which is reported to cause disassembly of caveolae and enclosed receptors found in caveolae (35, 36). Filipin was found to be effective in blocking the invasion of OmpA⁺ E. coli (E44) in a dose-dependent manner with a 50% inhibitory concentration of 2 μM and 80% inhibition at 4 μM (1.12 ± 0.03) × 10⁴ cfu/well for untreated HBMEC versus (0.2 ± 0.05) × 10⁴ cfu/well for 4 μM filipin; p < 0.001) (Fig. 1A). However, the total cell-associated bacteria (represented as binding) did not differ between untreated and filipin-treated cells, indicating that the inhibition was not due to inefficient binding of E. coli to HBMEC. In contrast, OmpA⁺ E. coli (E91), which did not show significant invasion in untreated cells, also showed no inhibition of either total cell-associated bacteria or background invasion in filipin-treated HBMEC. Similarly, pre-treatment of HBMEC with another inhibitor, cyclohextrin, an agent that inhibits caveola formation by sequestering plasma membrane cholesterol by inducing its efflux, also showed significant inhibitory effect on E. coli invasion. A dose of 2 mM exerted 40% inhibition, whereas a dose of 4 mM exerted a 75% inhibitory effect on invasion of HBMEC (1.0 ± 0.3) × 10⁴ cfu/well for untreated HBMEC versus (0.21 ± 0.10) × 10⁴ cfu/well for cyclohextrin-treated HBMEC; p < 0.001 (Fig. 1B) without significant differences in the total cell-associated bacteria. These results suggest that caveola may play a role in E. coli invasion.

Phosphorylated PKCα Binds to Caveolin-1 in E. coli Invasion—Having determined the importance of caveola formation in bacterial invasion, our next effort was to identify the activation of caveolin-1 during bacterial invasion. Because caveolin-1 activation requires interaction with phosphorylated PKCα, immunoprecipitation studies were performed using anti-PKCα antibody from total cell lysates of HBMEC infected with either E44 or E91. Western blot analysis of the immune complexes using anti-phospho-PKCα and anti-caveolin-1 antibodies indicated an association of caveolin-1 with phosphorylated PKCα only in HBMEC infected with E44 (invasive strain) and not in the cells infected with E91 (noninvasive strain) (Fig. 2A). The association of caveolin-1 with phosphorylated PKCα was observed within 5 min post-infection with E44 and peaked at 15 min, followed by a decline at 30 min. Densitometric analysis of caveolin-1 bands indicated a 3-fold increase in the association with phospho-PKCα when infected with E44 (Fig. 2B). In contrast, the noninvasive E. coli strain, despite containing similar levels of PKCα as revealed by blotting, showed neither phosphorylated species of PKCα nor caveolin-1. To rule out the possibility that the absence of caveolin-1 in E91 cell lysates is not due to low levels of caveolin-1, the total lysates of HBMEC infected with E44 and E91 were also immunoblotted with anti-caveolin-1 antibody. The immunoblot showed equal quantities of caveolin-1 in cell lysates. These results suggest that phosphorylated PKCα interacts with caveolin-1 in E. coli invasion of HBMEC.

Our previous studies showed that FAK activation is important for PKCα phosphorylation (22); thus, we next examined whether blocking FAK activation would inhibit caveolin-1 association with phospho-PKCα. Wild-type FAK- and FAK-related non-kinase (FRNK)-transfected HBMEC were infected with E44, and cell lysates were analyzed for caveolin-1 activation. FRNK has been shown to negatively regulate the function
down significant quantities of PKC and J were stained with both anti-caveolin-1. In a separate experiment, the monolayers of HBMEC infected with either E44 (A–D and I–L) or E91 (E–H and M–P) were fixed and stained with rhodamine-phalloidin (B and F) and anti-caveolin-1 antibody (C and G). Similarly, in a separate experiment, the monolayers were stained with both anti-caveolin-1 (J and N) and anti-phospho-PKCα (K and O) antibodies. Both green and red fluorescence was visualized using a dual-filter mode (D, H, L, and P). Arrows indicate the positions of bacteria, caveolin, actin, and phospho-PKCα.

of FAK (37). Our previous studies showed that overexpression of FRNK in HBMEC blocks E. coli cell-induced activation of FAK and subsequent actin rearrangements (20). As shown in Fig. 2C, immunoprecipitation with anti-PKCα antibody pulled down significant quantities of PKCs in both cell lysates. However, we observed significant phosphorylation of PKCα only in wild-type PFAK-transfected HBMEC, and the pattern was similar to that previously reported for non-transfected and infected HBMEC (22). When probed with anti-caveolin-1 antibody, the immune complexes showed profound association with caveolin-1 in these cells. However, in FRNK-transfected HBMEC, neither phospho-PKCα nor the corresponding association of caveolin-1 was observed, suggesting that the interaction of PKCα with caveolin-1 is downstream of FAK activation.

To confirm this sequence of events, we further examined the association of caveolin-1 with phospho-PKCα in cell lysates of HBMEC overexpressing a dominant-negative mutant form of PKCα. PKC/CAT-KR (a mutant in which the catalytic subunit of PKCα has been mutated) and wild-type PKC-transfected HBMEC were infected with E44 for various time points, and the cell lysates were analyzed. As expected, wild-type PKC-transfected HBMEC showed significant phosphorylation of PKCα between 10 and 15 min post-infection, whereas PKC/CAT-KR-transfected cells showed no activation at all (Fig. 2D). When stripped and reprobed with anti-caveolin-1 antibody, the blot showed association of caveolin-1 similar to that in non-transfected HBMEC, whereas there were no observable levels of associated caveolin-1 in PKC/CAT-KR-transfected HBMEC, suggesting that an intact and active PKCα is necessary for the association of caveolin-1. Lack of either phospho-PKCα or caveolin-1 was not due to unequal loading of the proteins, as the blot of cell lysates with anti-caveolin-1 antibody showed equal amounts of the protein. Taken together, these results suggest that E. coli cell-induced activation of caveolin-1 may be required for the formation of caveolae and is downstream of FAK and PKCα.

Localization of Caveolin-1 at the E. coli Entry Site with Actin and Phospho-PKCα—Because E. coli invasion of HBMEC appears to be mediated by caveolae, we next examined the distribution of caveolin-1 by immunocytocchemistry. Our previous studies showed that invading E. coli cells induce actin accumulation at the E. coli attachment site (23); thus, HBMEC infected with either E44 or E91 were fixed, permeabilized, and stained for both actin and caveolin-1. We observed several groups of bacteria attached to HBMEC, although the actin accumulation was observed only beneath select groups (Fig. 3, A and B). We have previously shown that only E. coli cells that are entering the cell elicit actin condensation, whereas those merely attached to the surface do not (23). Furthermore, the co-localization of caveolin-1 with actin at the bacterial entry site was also observed (Fig. 3, C and D). In contrast, such a pattern was not present in E91-infected cells (Fig. 3, E–H), as would be expected, because E91 does not activate the signals necessary for either actin accumulation or caveolin-1 activation. Because immunoprecipitation studies revealed that phospho-PKCα interacted with caveolin-1, we also stained the infected HBMEC with anti-phospho-PKCα and anti-caveolin-1 antibodies. Strong accumulation of caveolin-1 beneath the E. coli entry site was observed (Fig. 3J), with caveolin-1 co-localization with phospho-PKCα in E44-infected cells (Fig. 3, K and L), but not in E91-infected cells (Fig. 3, M–P). Taken together, these results suggest that activated PKCα is probably recruited to the plasma membrane to interact with caveolin-1 to initiate the formation of caveolae and pulls the bacterium into the cytoplasm utilizing the force of the actin network.

Transcellular Association of Caveolin-1 with E. coli in HBMEC—Transmission electron microscopy of E. coli invasion of HBMEC revealed that the bacterium resides in an endosome and crosses HBMEC with no signs of multiplication (23). In addition, we did not observe any lysosomal association with endosomes, indicating that E. coli cells somehow avoid lysosomal killing. The results described herein suggest that the en-
dosomal compartment might contain caveolin-1, which was previously shown to be responsible for avoiding lysosomal fusion (13). Thus, to confirm the presence of caveolin-1 during the transcellular process, we utilized the confocal laser microscopy Z section technique. HBMEC monolayers were infected with E44 for 30 min, fixed, permeabilized, and stained for caveolin-1 with anti-caveolin-1 antibody. Bacteria were stained with anti-K1 capsular polysaccharide antibody followed by fluorescent secondary antibody as described under “Results.” Optical sections were accumulated, and one section was analyzed for fluorescence scanning. Magnified images of bacteria stained in red (A), caveolin-1 stained in green (B), and an overlay of both A and B (C) are shown. The orthogonal projections of the optical section were viewed from XZ and YZ angles at a point where the two red lines intersect (D). The area of the section used for scanning is indicated by a blue line. The fluorescence intensities of red (E. coli) and green (caveolin-1) were plotted as a line scan (E) using MetaMorph Version 5.0 software.

antibody. We obtained 45 Z sections of 0.3 μm thickness from the top to the bottom of the cell; however, we have presented only five representative sections for each label. As shown in Fig. 4, several bacteria attached to HBMEC near the surface, and the corresponding caveolin-1 section revealed the distribution of caveolin-1 throughout the cells. A clear condensation of caveolin-1 around or beneath the bacteria in several places was observed (Fig. 4, A and F). Further sectioning showed that more bacteria were invading the cells especially at the center of monolayer. Interestingly, in this section, the density of caveolin-1 was slightly reduced throughout the cell, but more clear association was observed with the bacteria (Fig. 4, B and G). The sections that represent the middle of the cell (Fig. 4, D and I) showed a group of bacteria with significant association of caveolin-1. Other bacteria that showed accumulation of caveolin-1 around them at the top of the cell showed decreased association with the bacteria (Fig. 4, B and G). The sections that represent the middle of the cell (Fig. 4, D and I) showed a group of bacteria with significant association of caveolin-1. Other bacteria that showed accumulation of caveolin-1 around them at the top of the cell showed decreased association of caveolin-1, suggesting that these bacteria were still in the process of invasion at the plasma membrane. However, the bacteria at the center appeared to have already entered the cell. This group of bacteria continued to associate with caveolin-1 until reaching the bottom of the cell (Fig. 4, E–J). We also stained HBMEC infected with OmpA+ E. coli in a similar fashion, but did not observe any bacteria invading the cells and correspondingly no association of caveolin-1 (data not shown).

In addition, an optical section obtained from the middle of the cell infected with OmpA+ E. coli from a different experiment was analyzed for the co-localization of caveolin-1 with OmpA+ E. coli using MetaMorph imaging software. As shown in Fig. 5A, the optical section contained several bacteria inside the cell. The corresponding caveolin-1 staining showed accumulation of caveolin-1 around the bacteria, although discontin-
usually, but not in other areas where there were no bacteria (Fig. 5B). Overlay of these two images clearly showed that OmpA/E. coli and caveolin-1 were co-localized (Fig. 5C). Orthogonal sections of this optical slice also showed that E. coli (red) and caveolin-1 (green) were co-localized (yellow) when viewed from the area where several bacteria had invaded (Fig. 5D). Moreover, the fluorescence intensities of caveolin-1 and OmpA/E. coli were calculated from this optical section and converted into a line scan. The area of the section that was taken for these calculations is indicated with a blue line. In agreement with our above results that caveolin-1 accumulated at the bacterial entry site, we observed significant association of caveolin-1 (green) with E. coli (red) (peaks at positions 150–175, 210–225, and 250–260). These results strongly suggested that caveolin-1 accumulates around the invading E. coli cells and is probably present within the endosomal membranes that are formed via caveola.

Overexpression of a Dominant-negative Form of Caveolin-1 in HBMEC Blocks E. coli Invasion—Because caveolin-1 was found to be associated with phospho-PKCz, we decided to analyze the importance of this association in E. coli invasion. Phospho-PKCz is reported to bind to a specific region of caveolin-1 known as the scaffolding domain. Thus, HBMEC were transfected with a mutant form of caveolin-1 in which two amino acids responsible for phospho-PKCz interaction in the scaffolding domain (Cav−/HBMEC) were mutated. We also transfected HBMEC with wild-type caveolin-1 (Cav+/HBMEC) and pcDNA3 as positive and negative controls, respectively. Although efforts were initially directed toward stable transfection, we were unsuccessful in obtaining stable colonies. Thus, “éphémère (French for short-lived) transfections” were done at 30–40% confluence of HBMEC and continued in the presence of G418 until cultures were 90–100% confluent. For each experiment, a portion of the transfected cells were stained with anti-Myc antibody to assess the efficiency of transfection, and we found that 50–60% of the cells significantly expressed the Myc-tagged proteins (data not shown). Expression of mutant proteins was also verified by Western blot analysis for the expression of caveolin proteins. Total cell lysates of HBMEC and pcDNA3/HBMEC showed a band reactive to anti-caveolin-1 antibody, whereas Cav+/HBMEC and Cav−/HBMEC showed two bands (Fig. 6A). The upper band was the Myc-tagged caveolin-1, which migrated slightly slower than that of native caveolin-1. When probed with anti-Myc antibody, the same blot revealed one band in both Cav−/HBMEC and Cav+/HBMEC, suggesting that the caveolin-1 plasmid-transfected HBMEC express significant amounts of Myc-tagged caveolin-1. The equality of loading of total proteins in each lane was examined by blotting the cell lysates with anti-actin antibody. The equality of loading of total proteins in each lane was examined by blotting the cell lysates with anti-actin antibody. The equality of loading of total proteins in each lane was examined by blotting the cell lysates with anti-actin antibody. The equality of loading of total proteins in each lane was examined by blotting the cell lysates with anti-actin antibody. The equality of loading of total proteins in each lane was examined by blotting the cell lysates with anti-actin antibody. The equality of loading of total proteins in each lane was examined by blotting the cell lysates with anti-actin antibody. The equality of loading of total proteins in each lane was examined by blotting the cell lysates with anti-actin antibody. The equality of loading of total proteins in each lane was examined by blotting the cell lysates with anti-actin antibody. The equality of loading of total proteins in each lane was examined by blotting the cell lysates with anti-actin antibody. The equality of loading of total proteins in each lane was examined by blotting the cell lysates with anti-actin antibody. The equality of loading of total proteins in each lane was examined by blotting the cell lysates with anti-actin antibody. The equality of loading of total proteins in each lane was examined by blotting the cell lysates with anti-actin antibody. The equality of loading of total proteins in each lane was examined by blotting the cell lysates with anti-actin antibody. The equality of loading of total proteins in each lane was examined by blotting the cell lysates with anti-actin antibody.

FIG. 6. Inhibition of E. coli invasion and association of caveolin-1 with phospho-PKCz in HBMEC overexpressing a dominant-negative form of caveolin-1. A, total cell lysates (20 µg of protein) of HBMEC, pcDNA3/HBMEC (+/HBMEC), Cav−/HBMEC, and Cav+/HBMEC were blotted with anti-caveolin-1, anti-Myc, or anti-actin antibody. The arrow indicates Myc-tagged caveolin-1. B, confluent monolayers of various HBMEC were used for OmpA/E. coli invasion assays. In simultaneous experiments, the total cell-associated bacteria were also determined. The results are expressed as either relative binding or invasion, taking HBMEC values as 100%. The error bars represent the means ± S.D. of four separate experiments performed in triplicate. C, total cell lysates (20 µg of protein) of Cav−/HBMEC and Cav+/HBMEC infected with E44 for varying periods of time were subjected to the PepTag assay for PKCz activity. D, ~200 µg of total cell lysate proteins of Cav−/HBMEC and Cav+/HBMEC infected with E44 were immunoprecipitated (IP) with anti-phospho-PKCz antibody. The immune complexes were subjected to Western blotting (WB) with antiphospho-PKCz and anti-caveolin-1 antibodies. In addition, the total cell lysates (20 µg) were also subjected to immunoblotting with anti-caveolin-1 antibody.

To demonstrate that the decrease in the E. coli invasion of Cav−/HBMEC is due to the inability of phospho-PKCz to interact with caveolin-1, we first examined the activation of PKCz in these cells by a nonradioactive PepTag assay. Total cell lysates of both Cav−/HBMEC and Cav+/HBMEC showed significant activation of PKCz between 10 and 15 min post-infection with E44 (Fig. 6C), suggesting that overexpression of mutant caveolin-1 did not affect E. coli cell-induced PKCz activation. Next, we examined whether PKCz interacts with caveolin-1 by immunoprecipitation. Concomitant with the PepTag assay results, we observed significant amounts of phospho-PKCz in both Cav−/HBMEC and Cav+/HBMEC (Fig. 6D). However, no association of caveolin-1 was found in Cav−/HBMEC, whereas only Cav−/HBMEC showed considerable amounts of caveolin-1 coprecipitated with phospho-PKCz. When subjected to Western blotting with anti-caveolin-1 antibody, the total cell lysates of the transfected HBMEC indicated the presence of equal quantities of caveolin-1. This indicates that the absence of PKCz interaction with caveolin-1 might be responsible for the inhibition of E. coli invasion of HBMEC. In addition, this interaction could be crucial for the formation of caveolea.

Absence of Phospho-PKCz Interaction with Caveolin-1 at the Plasma Membrane in Cav−/HBMEC—Our previous studies showed that PKCz activated by invading E. coli cells translocates to the plasma membrane for further signaling events (22). One such event could be its interaction with caveolin-1, which is important for the formation of caveolea. Thus, we also examined whether the inhibition of E. coli invasion in Cav−/HBMEC is due to the inability of phospho-PKCz to interact with caveolin-1 at the plasma membrane. Membrane fractions of both Cav−/HBMEC and Cav+/HBMEC infected with E44 were prepared and assessed for the activation of PKCz by PepTag.
assay. As expected, both membrane fractions showed peak activation of PKCα at 15 min post-infection (Fig. 7A). The Cav+/HBMEC membrane fractions showed activation even at 5 min post-infection. The membrane fractions were then immunoprecipitated with anti-phospho-PKCα antibody, followed by immunoblotting with the same antibody and anti-caveolin-1 antibody. We observed that a greater amount of phospho-PKCα had been recruited to the membrane fraction in Cav+/HBMEC, with a corresponding increased interaction with caveolin-1 (Fig. 7B). Cav−/HBMEC showed normal levels of phospho-PKCα compared with non-transfected and infected HBMEC, but showed no association of caveolin-1. These results suggest that despite the translocation of activated PKCα to the plasma membrane, its inability to interact with caveolin-1 blocks E. coli invasion of Cav−/HBMEC.

Immunofluorescence studies on Cav+/HBMEC infected with OmpA− E. coli showed strong co-localization of caveolin at actin condensation sites (Fig. 8, A–D). In these cells, caveolin recruitment was much greater at the bacterial entry site compared with non-transfected HBMEC. Similarly, the density of PKCα recruitment was also much greater than in control cells, indicating that overexpression of caveolin may increase the interaction with PKCα, thus increasing the formation of more caveolae necessary for invasion. This could be the reason for slightly increased E. coli invasion of Cav+/HBMEC. In contrast, in Cav−/HBMEC, although there was significant phospho-PKCα and actin accumulation beneath the bacteria, there was no detectable accumulation of caveolin-1 (Fig. 8, I–P). Thus, it is clear that an intact scaffolding domain contributes to the association of caveolin-1 with PKCα, thereby facilitating bacterial invasion.

**Cell-permeable Scaffolding Peptide of Caveolin-1 Blocks E. coli Invasion**—Because the binding of PKCα to the scaffolding peptide of caveolin-1 has been found to be crucial to the internalization of caveolae, we used a peptide that represents the caveolin-1 scaffolding domain (amino acid residues 82–101) and a scrambled peptide (control) to examine their effect on E. coli invasion. These peptides were synthesized with a 16-amino acid portion of the AP homeodomain (RQIKIWFQNR-RMKWKK), a Drosophila transcription factor, at the N-termi-
Fig. 9. Inhibition of *E. coli* invasion of HBMEC by a cell-permeable peptide that represents the scaffolding domain. Fluoresceinated AP-Cav and AP-Cav-X peptides (4 μM) were incubated with confluent monolayers of HBMEC for 6 h, washed, and viewed under transmitted light with a blue filter (A) or fluorescence (B). Only AP-Cav peptide-treated cells are shown. Confluent monolayers of HBMEC were treated with various concentrations of either AP-Cav or AP-Cav-X peptide as described under “Experimental Procedures,” washed, and used for *E. coli* invasion assays (C). In duplicate experiments, the total cell-associated bacteria (represented as binding) were determined. The peptide-treated and E44-infected HBMEC lysates were subjected to immunoprecipitation (IP) studies with anti-phospho-PKCα antibody. The immune complexes were probed with either anti-phospho-PKCα or anti-caveolin-1 antibody. In addition, total cell lysates were also subjected to Western blotting (WB) with anti-caveolin-1 antibody to verify the presence of caveolin-1 (D).

nal side of both the scaffolding peptide (AP-Cav) and the scrambled peptide (AP-Cav-X). The AP protein facilitates homogeneous uptake of peptides or oligonucleotides into cultured mammalian cells through a non-endocytic and non-degradative pathway (34). To analyze the efficiency of entry of the peptides into the cell, the peptides were labeled with fluoresceinamine and then added to the cells. These fluoresceinated peptides entered 60–70% of the cells at a 2 μM concentration within 6 h of incubation and distributed throughout the cell (Fig. 9, A and B). HBMEC pretreated with AP-Cav showed significant inhibition of *E. coli* invasion compared with HBMEC pretreated with AP-Cav-X in a dose-dependent manner ((0.4 ± 0.2) × 10⁴ cfu/well for AP-Cav versus (1.1 ± 0.3) × 10⁴ cfu/well for AP-Cav-X at 4 μM; p < 0.01) (Fig. 9C). In contrast, the total cell-associated bacteria did not differ significantly between AP-Cav- and AP-Cav-X-treated HBMEC, suggesting that lack of invasion is not due to the inability of bacteria to bind to the cells. Consistent with the ability of AP-Cav to inhibit the *E. coli* invasion, immunoprecipitation of the cell lysates of HBMEC treated with the scaffolding peptide with anti-phospho-PKCα antibody resulted in the inhibition of caveolin-1 association with phospho-PKCα (Fig. 9D). In contrast, AP-Cav-X-pretreated HBMEC showed an association of caveolin-1 with phospho-PKCα similar to that in untreated HBMEC. Lack of caveolin-1 association with phospho-PKCα is not due to the absence of caveolin-1 in these fractions, as the total cell lysates showed equal quantities of caveolin-1 when immunoblotted with anti-caveolin-1 antibody. In addition, we also examined the association of actin and phospho-PKCα by immunocytochemistry. The results were similar to those obtained for Cav–/HBMEC and Cav+/HBMEC (similar to Fig. 8), in which we observed accumulation of actin and phospho-PKCα at the sites of *E. coli* entry, but not co-localization of caveolin-1 in AP-Cav-pre- treated HBMEC. This confirms our hypothesis that *E. coli* cells use caveolae as a mode of entry into HBMEC, thereby probably ensuring their survival by avoiding fusion with lysosomes.

**DISCUSSION**

*E. coli* invasion of HBMEC occurs by a zipper-like mechanism in which the host cell plasma membrane enwraps the invading bacteria and becomes an endosome (23). This mechanism requires *E. coli* cell-induced HBMEC cytoskeletal rearrangements for the accumulation of actin at the site of bacterial entry. Transcytosis of *E. coli* in the endosome occurs within 30 min post-infection without multiplication. In addition, there is no evidence of bacterial killing inside the endosome, which could be due to the ability of bacteria within the endosome to avoid the fusion of lysosomes. The nature of this endosome is not known to date. A few studies have attempted to establish the role of caveolae in the entry of pathogens such as *E. coli* expressing FimH and *Chlamydia* (1, 2, 27). However, the molecular events leading to the formation of caveolae for any pathogenic microorganism have not been described thus far.

Our findings underscore the critical nature of caveola formation in *E. coli* invasion of HBMEC via interaction of caveolin-1 with phospho-PKCα. In addition, these data indicate that the integrity of cholesterol-enriched microdomains that are normally present in HBMEC must be necessary for invasion. This may explain why the drugs filipin and cyclodextrin, which are known to specifically disrupt raft microdomains, inhibit most *E. coli* entry. Because our previous studies showed that *E. coli* OmpA interacts with a 95-kDa gp96 like molecule for invasion of HBMEC (19), it is possible that the OmpA receptor might be enriched in caveolae during the invasion process. In agreement with this concept, we observed clustering of the OmpA receptor beneath the *E. coli* entry site by immunocytochemistry by 15 min post-infection. Receptors for insulin have been shown to cluster within caveolae and to interact with caveolin-1 to differentially modulate post-receptor signaling (33). Thus, it is reasonable to speculate that OmpA interacts with its receptor, gp96, which in turn interacts with caveolin-1 and clusters within the caveolae for further signaling necessary for *E. coli* invasion. However, caveolae are small relative to *E. coli* to be transported across the cell; therefore, it would be possible that several OmpA molecules on *E. coli* induce multiple raft domains after interaction with HBMEC receptors and that these are subsequently united to surround the bacterium.

Our studies show that *E. coli* invasion of HBMEC induces activation of PKCs by three folds in an OmpA-dependent manner (22). Activated PKCα then translocates to the plasma membrane for further signaling events. A major finding of this study is that the activated PKCs interact with caveolin-1, a specific marker of caveolae. We also found by immunocytochemistry that PKCα activated by invading *E. coli* cells co-localizes with caveolin-1. In addition, these two molecules are also present at the actin condensation sites beneath *E. coli* attachment, reflecting possible signaling complex formation around the caveolae for efficient transcytosis of *E. coli*. However, the role of caveolin-1 in further signaling events is not clear at this point. Interestingly, overexpression of dominant-negative forms of either FAK (FRNK) or PKCα (PKO/CAT-KR) inhibited the association of phospho-PKCα with caveolin-1, indicating that both FAK and PKCα may be upstream of caveolin-1 interaction. Thus, targeting of phospho-PKCα to caveolae could be an important event in *E. coli* invasion. Several studies have previously shown that PKCα regulates the membrane invaginations and is enriched in caveolae, and our results are in agreement with these observations (4, 25).

We further demonstrated that phospho-PKCα interacts at the scaffolding domain of caveolin-1, as overexpression of a dominant-negative form of caveolin-1 in which the amino acids in the scaffolding domain have been mutated significantly blocked *E. coli* invasion of HBMEC. Consistent with these
Indeed, studying the scaffolding domain-mediated signaling pathways. Inhibition of acetylcholine-induced vasodilation and nitric oxide production takes place in the BBB. The immunoprecipitation studies in which phospho-PKC interact with caveolin-1 at the bacterial entry site despite the recruitment of phospho-PKC to the membrane. These results are in agreement with the immunoprecipitation studies in which phospho-PKCα present in the membrane fractions of Cav−/HBMEC showed no association with caveolin-1. These observations suggest that phospho-PKCα recruitment to the E. coli entry site does not require its interaction with caveolin-1, but is absolutely necessary for efficient E. coli invasion. Similarly, we observed actin accumulation at the bacterial attachment site despite absence of invasion. Because our previous studies showed that PKCα activation is important for actin accumulation beneath the bacteria (22), PKCα might play two differential roles in E. coli invasion: one in actin rearrangements and the other in caveolin-1 activation and effective caveola internalization. These results are in sharp contrast to those observed for SV40 internalization via caveola, in which either actin condensation or tyrosine phosphorylation occurs only after SV40-induced caveola formation (31). As a control, we also used wild-type caveolin-1-transfected HBMEC in E. coli invasion assays, which showed a slight increase in invasion. Previous studies by other investigators have shown that overexpression of caveolin-1 induces the formation of caveolae in several cell types (3, 5, 13). Thus, the increase in E. coli invasion could be due to nonspecific trapping of bacteria in caveolae. Alternatively, overexpression of caveolin-1 increases the turnover of receptors to the cell surface, and these receptors are subsequently responsible for internalization of more E. coli. The survival of E. coli in the endothelium depends on the ability of the intracellular parasite to resist endosomal acidification and lysosomal fusion. Our studies show that during transcytosis, E. coli cells remain enclosed in the endosome containing caveolin-1, suggesting that caveola-mediated entry may play an important role in preventing lysosomal fusion. The caveola-mediated SV40 and C. trachomatis entry pathways transport the microorganism to the endoplasmic reticulum, a normal target for endocytic cargo, rather than the endosomal/lysosomal compartment (30–32). However, caveolin-1 may not be the sole molecule responsible for the avoidance of lysosomal fusion, as shown for Chlamydia psittaci, in which early gene expression by the bacterium in the endosome is important (38). Despite poor understanding of the mechanism that lead to lysosomal avoidance, the continuing association of caveolin with E. coli in caveola-mediated entry may have important implications in the crossing of the BBB.

In summary, we have shown that E. coli internalization of HBMEC occurs via caveola. The scaffolding domain of caveolin-1 interacts with PKCα at the plasma membrane beneath the E. coli entry site. Because invasion by E. coli depends on OmpA interaction with its receptor, a gp96-like protein, identification of the role of caveolin-1 in bringing these receptor molecules into caveola furthers our understanding of the mechanism of invasion.

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