Modulation of Myosin Light-Chain Phosphorylation by p21-Activated Kinase 1 in *Escherichia coli* Invasion of Human Brain Microvascular Endothelial Cells

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Cytoskeletal dynamics, modulated by actin-myosin interactions, play an important role in *Escherichia coli* K1 invasion of human brain microvascular endothelial cells (HBMEC). Herein, we show that inhibitors of myosin function, butanedione monoxide and ML-7, significantly blocked the *E. coli* invasion of HBMEC. The invasive *E. coli* induces myosin light-chain (MLC) phosphorylation during the invasion process, which gets recruited to the site of actin condensation beneath the bacteria. We also show that invading *E. coli* downregulates the activity of p21-activated kinase 1 (PAK1), which is an upstream regulator of MLC kinase (MLCK). Overexpression of wild-type PAK1 and constitutively active PAK1 in HBMEC inhibits *E. coli* invasion significantly with a concomitant decrease in MLC phosphorylation. The inhibition of *E. coli* invasion by these PAK1 mutants is due to the absence of phospho-MLC at the actin condensation points. In contrast, the dominant-negative PAK1 shows no effect either on the invasion or on MLC phosphorylation or phospho-MLC recruitment to the actin focal points, suggesting that activated PAK1 inactivates MLCK. Taken together, these results suggest that *E. coli* invasion of HBMEC induces MLC phosphorylation by inhibiting the activity of PAK1 and the recruitment of phosphorylated MLC to the site of actin condensation beneath the bacteria for efficient internalization of *E. coli* into HBMEC.

The strategies adapted by a diverse group of intracellular microorganisms to induce cytoskeletal changes for their own uptake often involve a very sophisticated subversion of host cellular function; however, these strategies are all distinctly different. The *Escherichia coli* K1, which causes meningitis in neonates, is an example of an intracellular pathogen that induces actin reorganization to invade human brain microvascular endothelial cells (HBMEC). The remodeling of actin induced by *E. coli* occurs in an outer membrane protein A (OmpA)-dependent interaction with a 95-kDa receptor specifically expressed on HBMEC (18). In response to this interaction, invading *E. coli* induces the increased phosphorylation of focal adhesion kinase (FAK) and paxillin, a protein that associates with actin (22). Our studies further showed that autophosphorylation of FAK is crucial for its activation and that the overexpression of a dominant-negative form of FAK, in which the autophosphorylation site is mutated, significantly blocked the invasion. In addition, we have shown that the activation and interaction of phosphatidylinositol 3-kinase (PI 3-kinase) with activated FAK is important for the invasion process (23). Another cellular response stimulated by invading *E. coli* is the activation of protein kinase C- α (PKC- α), which translocates to the plasma membrane (27). The activated PKC- α further interacts with its substrate MARCKS, which is thought to be relieved from its interaction with actin so that the

actin filaments can accumulate at the bacterial entry site. In agreement with this concept, overexpression of a dominant-negative form of PKC- α in HBMEC significantly blocked the accumulation of actin beneath the bacterial entry site, which in turn blocked the *E. coli* invasion of HBMEC by more than 80%. The activated PKC- α at the plasma membrane also interacts with caveolin-1, a specific marker of caveolae, to trigger the formation of caveolae in which the *E. coli* are traversed across the HBMEC (28).

The interaction of actin and myosin, regulated by myosin light chain (MLC), primarily modulate cytoskeletal dynamics. Although the role of actin in *E. coli* invasion is clearly established, nothing is known about the role of myosin and its upstream regulators. Phosphorylation of Ser¹⁹ of the regulatory MLC stimulates the actin-activated ATPase activity of myosin II and regulates the force generating ability of myosin II in vivo (8, 30). MLC phosphorylation is regulated by the balance of two enzymatic activities, i.e., MLC kinase (MLCK) and myosin phosphatase. MLCK is regulated by Ca²⁺-dependent calmodulin and is believed to be a major kinase in both smooth muscle and nonmuscle cells. MLCK is a target of the Rho family of GTPases in signaling to the cytoskeleton. MLCK phosphorylation by p21-activated kinase 1 (PAK1) is associated with inhibition of MLCK activity and decreased MLC phosphorylation (5, 10, 24). The PAK family of serine/threonine kinases comprises at least four isoforms that are differentially expressed in mammalian cells (12, 13). PAK1 was initially identified as a Rac1-binding protein and was further shown to interact significantly with the GTP-bound forms of Rac1 and Cdc42 (3, 5, 12). The catalytic activity of PAK1 is

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regulated by the binding of Rac1 or Cdc42 to a highly conserved motif in the N terminus, known as the p21-binding domain or Cdc42/Rac interactive binding domain (1, 16, 17). The binding of Rac/Cdc42 induces a conformational change in PAK1, which is thought to be necessary for autophosphorylation at several sites and for enabling the phosphorylation of exogenous substrates (5). Interestingly, PAK1 has also been shown to phosphorylate MLC directly in mammalian fibroblasts (25).

The bacterial pathogen *Salmonella enterica* serovar Typhimurium, which colonizes animals, has been shown to require PAK1 activation for the induction of nuclear responses in host cells but not for actin rearrangement (2). The stimulation of nuclear responses via PAK1 requires the function of Cdc42. However, Criss et al. further showed that Rac1, but not Cdc42, was activated during bacterial entry at the apical plasma membrane in polarized cells (4). We report here that *E. coli* invasion of HBMEC increased the phosphorylation of MLC in host cells and its recruitment to the sites of actin accumulation beneath the bacteria. We also found that MLC phosphorylation is associated with decreased activation of PAK1. These results are the first to show that the modulation of MLC phosphorylation, by downregulating the PAK1 activity, is required for invasion of bacterial pathogens thus far examined.

MATERIALS AND METHODS

Bacterial strains, cell lines, and culture conditions. The *E. coli* strains E44 and E91 used in the present study are derivatives of the *E. coli* K1 strain RS218 (serotype O18:K1:H7). Strain E44 is a spontaneous rifampin-resistant and invasive mutant, whereas E91 is a noninvasive mutant lacking the *ompA* gene that was used as a negative control (19, 20). The strains were grown in brain heart infusion medium supplemented with the antibiotics rifampin (100 µg/ml) for E44 and tetracycline (12.5 µg/ml) for E91. HBMEC were grown in medium containing M199–Ham F-12 (1:1) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, and 2 mM glutamine and then cultivated in a cell culture incubator at 37°C in an atmosphere containing 5% CO₂ (26).

Transfection of HBMEC with the mutants of PAK1. All PAK1 variants, human wild-type PAK1 (Wt-PAK1), constitutively active PAK1 (cAc-PAK1; H83L and H86L), and dominant-negative PAK1 (DN-PAK1; H83L, H86L, and K299A) were inserted into pCMV6M with a Myc epitope at the amino terminus for detection (5, 6). HBMEC were transfected with mammalian expression vectors by using Lipofectamine and propagated as described previously (27).

Antibodies. Anti-phospho-MLC (specific to Thr-18/Ser-19), anti-FAK, and anti-Myc antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.). Anti-MLC antibody was obtained from Sigma (St. Louis, Mo.). Anti-phosphotyrosine antibody (4G10) was from Upstate Biotechnology, Inc. (Waltham, Mass.). Anti-PAK1 and anti-phospho-PAK1 (Thr-423)/PAK2 (Thr-402) were from Cell Signaling (Beverly, Mass.). Anti-phospho-MLC antibody (pp2b) that specifically recognizes Ser-19 was kindly provided by Fumio Matsumura (Rutgers University, Piscataway, N.J.).

***E. coli* invasion assays.** Entry of bacteria into HBMEC was quantified by the standard gentamicin protection assay as described previously (19). Briefly, confluent monolayers of HBMEC in 24-well plates were infected with 10⁷ CFU of *E. coli* strains (multiplicity of infection of 100) and incubated for 90 min at 37°C in an atmosphere containing 5% CO₂. The cells were washed thoroughly with RPMI 1640 and further incubated with gentamicin (100 µg/ml) in infection medium for 1 h. The cells were then washed extensively with RPMI 1640 and lysed with 0.5% of Triton X-100. The intracellular bacteria were enumerated by plating on 5% sheep blood agar. For inhibition studies, stock solutions of 2,3-butanedione monoxime (BDM) and ML-7 were made in dimethyl sulfoxide (DMSO) and diluted in infection medium. These inhibitors were preincubated with the BMEC monolayers for 30 min at 37°C before the addition of bacteria and were present throughout the invasion period.

Preparation of cell lysates, immunoprecipitations, and Western blot analysis. Confluent cell cultures in 100-mm-diameter dishes were incubated with either E44 or E91 for various periods of time at 37°C. The cells were then rinsed with ice-cold phosphate-buffered saline (PBS) and scraped off into 500 µl of radio-

immunoprecipitation assay buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 10 µg of phenylmethylsulfonyl fluoride/ml, 2 mM aprotinin, 1 mM sodium orthovanadate). The cells were disrupted mechanically by sonication, followed by centrifugation for 10 min at 5,000 × *g* to remove cell debris. The supernatants were used as cell lysates. For immunoprecipitations, the cell lysates (300 µg of proteins) were incubated with the respective antibody at 4°C overnight. The immune complexes were incubated with protein A-agarose beads for 1 h at 4°C, washed three times in ice-cold lysis buffer, and boiled in 30 µl of SDS-polyacrylamide gel electrophoresis sample buffer. For Western analysis, ca. 30 µg of total cell lysates were separated by electrophoresis on an SDS–12% polyacrylamide gel, and the proteins were transferred to nitrocellulose. The blots were blocked in blocking buffer (100 mM Tris-Cl [pH 7.5], 150 mM NaCl, 0.1% Tween 20, 3.0% bovine serum albumin or 5% nonfat milk) and then probed with the appropriate antibody. The same blot was stripped by using a stripping solution (Pierce Co.) and reblocked for subsequent probing with additional antibodies. The immunoreactive proteins were detected by using a chemiluminescence detection kit (Super Signal Chemiluminescence kit; Pierce Co.). The protein bands on the X-ray films were quantitated on a Bio-Rad densitometer by using Alpha-Imager software (Alpha Innotech Corp., San Francisco, Calif.).

PepTag assay. The PepTag assay for PKC-α utilizes brightly colored, fluorescent peptide substrates that are highly specific for PKC-α (Promega). The hot pink color is imparted by the addition of a dye molecule to the PepTag peptide substrate. Phosphorylation by PKC-α of their specific substrate alters the peptide's net charge from +1 to −1. This change in the net charge of the substrate allows the phosphorylated and nonphosphorylated versions of the substrate to be rapidly separated on a 1% agarose gel. The phosphorylated species migrates toward the positive electrode, whereas the nonphosphorylated substrate migrates toward the negative electrode. The lysates of HBMEC infected with E44 were subjected to PepTag assays as described previously (27).

Immunofluorescence. Cells were grown in eight-well chamber slides (LabTek) and were treated with either E44 or E91 for various time periods. The bacteria were removed by aspiration; the cells were washed with PBS three times and were fixed in 2% paraformaldehyde for 15 min, followed by two washes with PBS. After fixation, slides were rehydrated with three washes in PBS before a 30-min incubation in blocking buffer (1% bovine serum albumin and 0.5% Triton X-100 in PBS). Primary antibodies were diluted in blocking buffer (p-MLC antibody [1:100]), and the slides were incubated at room temperature for 1 h. Slides were washed three times for 5 min in PBS and then were incubated 45 min with an anti-goat fluorescein isothiocyanate-labeled secondary antibody (1:250 dilution) for p-MLC and rhodamine-phalloidin for actin staining. Slides were given three washes in PBS, air dried, and mounted with Vectashield containing DAPI (4',6'-diamidino-2-phenylindole; Vector Laboratories). Cells were viewed by a Leica (Wetzlar, Germany) DMRA microscope with Plan-apochromat 40×/1.25 NA and 63×/1.40 NA oil immersion objective lenses. Image acquisition was with a SkyVision-2/VDS digital charge-coupled device (12-bit, 1,280 × 1,024 pixels) camera in unbinned or 2×2 binned models into EasyFISH software, saved as 16-bit monochrome and merged as 24-bit RGB TIFF images (Applied Spectral Imaging, Inc., Carlsbad, Calif.).

RESULTS

Inhibitors of myosin activity block the *E. coli* invasion of HBMEC. Our previous studies showed that cytoskeletal reorganization occurs during *E. coli* invasion of HBMEC (21). Myosin is an important component of the cell cytoskeleton thus, we set out to examine the role of myosin in *E. coli* invasion by employing inhibitors that affect myosin function. HBMEC were pretreated with BDM, which inhibits the ATPase activity of nonmuscle myosin II, before performing the *E. coli* invasion assays. As shown in Fig. 1A, BDM treatment blocked *E. coli* invasion in a dose dependent manner with a 55% inhibitory effect at a 10 mM concentration and ca. 75% inhibition at a 25 mM concentration (9,200 ± 495 CFU for untreated HBMEC versus 3,000 ± 375 CFU for BDM-treated HBMEC; *P* < 0.01). However, the total cell-associated bacteria between treated and untreated cells did not differ significantly, suggesting that lack of invasion could not be due to inefficient binding of the bacteria. The noninvasive *E. coli*, in

FIG. 1. Effect of inhibitors of myosin function on *E. coli* invasion of HBMEC. Different concentrations of BDM (A) or ML-7 (B) were added to confluent monolayers of HBMEC for 30 min at 37°C prior to the addition of OmpA⁺ *E. coli* and invasion assays carried out. OmpA⁻ *E. coli*, E91, was used as a negative control in pcDNA3. DMSO, in which the inhibitors were dissolved, was used as a solvent control. The invasion of *E. coli* in the presence of these inhibitors was compared with untreated cells (control) taken as 100% invasion. The experiments were performed at least three times in triplicate and are expressed as means $\pm$ the standard deviations. The invasion of *E. coli* at highest concentrations of these inhibitors is significantly inhibited compared to the control ($P < 0.01$ [unpaired two-tailed *t* test]).

contrast, was able to bind HBMEC in the presence of BDM but, as expected, did not invade in control cells.

In addition, we examined the effect of another myosin inhibitor, ML-7, which inhibits the function of MLCK, an enzyme mainly responsible for the phosphorylation of MLC. *E. coli* invasion of HBMEC pretreated with ML-7 was significantly blocked compared to untreated HBMEC (Fig. 1B). We observed a 50% inhibition of *E. coli* invasion at a concentration of 2 μ M ML-7 concentration, whereas a 68% inhibition was achieved at a concentration of 4 μ M (9,375 $\pm$ 530 CFU for control HBMEC versus 3,350 $\pm$ 290 CFU for ML-7-treated HBMEC; $P < 0.01$). The total cell-associated bacteria was not significantly different between ML-7-treated and untreated cells ($1.12 \times 10^5 \pm 0.11 \times 10^5$ CFU/well for ML-7 versus $0.95 \times 10^5 \pm 0.32 \times 10^5$ CFU/well for the control). Interestingly, the concentrations of ML-7 used in the present study are far less than the concentrations used by other investigators for a variety of other cells, which range up to 50 μ M. Further increases in the concentrations of these inhibitors had deleterious effects on the HBMEC monolayers. The effects of these inhibitors on both HBMEC and bacteria were assessed by trypan blue dye exclusion and colony count methods, respectively (19). The results show no significant effect on the viability of both HBMEC and bacteria under the conditions employed (data not shown). Taken to-

gether, these results suggest that myosin plays a role in *E. coli* invasion that is most likely due to modulation of the phosphorylation status of MLC by MLCK.

Invasion of *E. coli* induces MLC phosphorylation in HBMEC. We next examined whether *E. coli* induces MLC phosphorylation during invasion by subjecting the HBMEC cell lysates to immunoblotting with an anti-pMLC antibody. The invasive *E. coli*, E44, induced the phosphorylation of MLC at 10 min postinfection, which peaked at 15 min, followed by a decline by 30 min postinfection (Fig. 2A). In contrast, the noninvasive *E. coli*, E91, did not induce such an increase in phosphorylation. Densitometric scanning of pMLC bands showed a three- to fourfold increase at 15 min compared to control cells and E91-treated HBMEC (Fig. 2B). The blot when stripped and reprobed with anti-MLC antibody showed similar levels of proteins in each lane. These data show that invasive *E. coli* induces a time-dependent phosphorylation of MLC in HBMEC. The time course of increased MLC phos-

FIG. 2. Phosphorylation of MLC in *E. coli* invasion. (A) Confluent monolayers of HBMEC were treated with either OmpA⁺ *E. coli* (E44) or OmpA⁻ (E91) *E. coli* for various periods of time as indicated; the total cell lysates were prepared and subjected to Western analysis with either anti-phospho-MLC (pMLC) or anti-MLC antibodies. (B) The density of both pMLC and MLC bands were estimated by a densitometer and showed as the areas of the bands. The measurement of the band intensity was linear up to 100,000 U since it was calibrated with an anti-actin antibody blot. (C) In some experiments, the monolayers were pretreated with ML-7 (10 μ M) prior to incubation with OmpA⁺ *E. coli* (E44+ML-7). (D) In other experiments, the pMLC antibody was preincubated with pMLC peptide (10 μ g) before being used for Western blotting (pMLC+Ab+pMLC peptide). In addition, the pMLC blot was stripped and reprobed with anti-MLC antibody.

phorylation correlates with the changes in actin rearrangements, which we have previously reported with a peak accumulation occurring between 15 and 20 min postinfection with E44 (23).

The key enzyme that phosphorylates MLC is MLCK; thus, ML-7 inhibition of *E. coli* invasion could be due to the blocking of MLCK activity. However, PAKs, especially PAK1, have also been shown to directly phosphorylate MLC on Ser-19 (25). Therefore, to ensure that ML-7 inhibition is due to MLCK inhibition, HBMEC were pretreated with ML-7 prior to *E. coli* infection. Immunoblotting with anti-pMLC antibody, as expected, showed significant inhibition of MLC phosphorylation in ML-7-treated cells compared to DMSO-treated HBMEC (Fig. 2C). We also carried out immunoblotting of cell lysates of HBMEC by using anti-pMLC antibody preincubated with phospho-MLC peptide to examine the specificity of the antibody reactivity. Blocking of the binding site of pMLC antibody with the peptide inhibited its reactivity to pMLC induced by *E. coli* invasion, suggesting that the antibody specifically recognizes pMLC (Fig. 2D). The absence of pMLC antibody reactivity is not due to lack of proteins on the blot since the blot reprobed with anti-MLC antibody showed significant amounts of proteins. Taken together, these results suggest that invasive *E. coli* induce MLC phosphorylation very rapidly, probably via MLCK.

Phosphorylated MLC is recruited to actin accumulation sites beneath the *E. coli*. We next attempted to examine the localization of phosphorylated MLC by immunofluorescence after infection of cells with E44. Since our previous studies showed that actin accumulates beneath the bacterial entry site, we also stained the infected HBMEC with rhodamine-phalloidin, which binds actin filaments. As shown in Fig. 3, we observed stress fibers at the apical surface of the untreated HBMEC, whereas punctate staining was observed with anti-pMLC antibody throughout the cell (Fig. 3A to D). HBMEC infected with E44 showed several groups of bacteria attached to the surface and condensation of actin beneath a few groups of bacteria (Fig. 3F). Our previous studies showed that only bacteria that are in the process of invasion elicit actin condensation as opposed to simply adherent bacteria (21, 27, 28). In addition, only one or two bacteria appear to be entering the cell despite the presence of a group, which could be due to competition for the receptor structure necessary for invasion. Interestingly, a dense accumulation of pMLC at the bacterial entry site was observed in these cells (Fig. 3G). Dual fluorescence image of these cells clearly showed that pMLC was colocalized with actin condensation (Fig. 3F, yellow color). The noninvasive *E. coli*, in contrast, could not elicit accumulation of either actin or pMLC beneath the bacteria (Fig. 3I to L). To further confirm that the inhibitory effect of ML-7 on *E. coli* invasion is due to the absence of pMLC at the actin condensation sites, HBMEC monolayers were pretreated with ML-7 inhibitor (10 μ M) as described above and then infected with E44. Both actin accumulation and the pMLC recruitment were completely inhibited by pretreatment with ML-7 (Fig. 3M to P). However, stress fiber formation was not significantly affected by this treatment. Again, lack of pMLC antibody reactivity in ML-7-treated cells shows the specificity of the pMLC antibody. Taken together, these results suggest that invasive

E. coli induces phosphorylation of MLC, which subsequently is recruited to the site of bacterial entry along with actin.

Downregulation of PAK1 activity in *E. coli* invasion increases MLC phosphorylation. Since MLC phosphorylation was inhibited by ML-7, it is likely that MLCK plays a central role in the phosphorylation of MLC. Thus, it is of interest to examine the modulation of MLCK activity in *E. coli* invasion. MLCK, a downstream target for PAK1, is inactivated upon phosphorylation by PAK1. Therefore, we examined PAK1 activity as an indicator of MLCK activity. Total cell lysates of HBMEC treated with E44 and E91 for various periods of time were subjected to Western blotting with anti-phospho-PAK1/PAK2 antibody (anti-PAK1 antibody is not available to date) which recognizes both the phospho-threonine 423 (autophosphorylation site) in the kinase domain of PAK1 (62 kDa) and threonine 402 of PAK2 (58 kDa). As shown in Fig. 4A, the phosphorylation of PAK1 decreased significantly between 10 and 15 min postinfection with E44, whereas no changes were observed in cell lysates infected with E91. The phosphorylation of PAK2, however, did not change at any time points in these lysates. The blot when stripped and reprobed with anti-PAK1 antibody (specific to PAK1 only) showed equal quantities of proteins in all of the lanes and matched with the upper band in phospho-PAK blot. Densitometric scanning of the phospho-PAK1 bands showed a threefold decrease in the intensity of the band at 15 min compared to uninfected cells (Fig. 4B). In contrast, E91-treated HBMEC lysates showed no decrease in PAK1 phosphorylation. These results clearly suggest that the invasive *E. coli* induces downregulation of PAK1 activity, which might result in increased MLCK activity and increased phosphorylation of MLC.

Overexpression of wild-type and cAc-PAK1 inhibits *E. coli* invasion, whereas DN-PAK1 has no effect. To further investigate the role of PAK1 on MLC phosphorylation and in *E. coli* invasion, HBMEC transfected with either DN-PAK1, cAc-PAK1, or Wt-PAK1 constructs were used. DN-PAK1 contains mutations in both the kinase domain (R299) and the GTPase-binding domain (H83L and H86L) (Fig. 5A). Previous studies showed that DN-PAK1 interferes with the function of endogenous PAK1, presumably by associating with substrates or other PAK-interacting proteins (11). The cAc-PAK1 (H83L and H86L), however, is deficient in binding to Rac1 and has enhanced kinase activity (6). PAK1 mutant cDNAs were cloned into a mammalian expression vector containing Myc epitope. The expression of PAK1 proteins was then assessed in the HBMEC transfectants by immunoblotting with anti-Myc antibody of the cell lysates. The blots showed significant levels of PAKs in Wt, DN, and cAc-transfected HBMEC but not in pcDNA3/HBMEC (Fig. 5B). Interestingly, E44 invasion was significantly reduced by >70% in both Wt-PAK1/HBMEC and cAc-PAK1/HBMEC ($15,935 \pm 1,500$ CFU for pcDNA3/HBMEC versus $\sim 5,000 \pm 370$ CFU for Wt-PAK1/HBMEC or cAc-PAK1/HBMEC; $P < 0.01$) (Fig. 5C). In contrast, DN-PAK1/HBMEC showed levels of invasion similar to those of pcDNA3/HBMEC. These results indicate that Wt- and cAc-PAK1-transfected HBMEC contain significant amounts of PAK1 in an active state, thereby facilitating increased phosphorylation of MLCK (less activated), which leads to a reduction in MLC phosphorylation. However, in DN-PAK1/HBMEC the opposite scenario appears to occur.

FIG. 3. Recruitment of pMLC to actin condensation sites beneath *E. coli* entry and its inhibition by ML-7. (A to D) HBMEC, nontransfected and uninfected, was used as a control to show the normal pattern of actin and pMLC staining. Confluent monolayers of HBMEC were infected with either OmpA⁺ *E. coli* (E to H) or OmpA⁻ *E. coli* (I to L) for 15 min, followed by a thorough washing and fixation with 2% paraformaldehyde. (M to P) In some experiments, the monolayers were pretreated with ML-7 (10 μ M) before they were infected with OmpA⁺ *E. coli*. The monolayers were stained with either rhodamine-phalloidin (B, F, J, and N) or anti-pMLC antibody (C, G, K, and O) and mounted by using anti-fade solution with DAPI. The bacteria were visualized by using transmitted light optics with a blue filter (A, E, I, and M). The cells were also visualized with dual filter mode for both red and green fluorescence (D, H, L, and P). The confocal images were assembled and labeled by using Adobe Photoshop 6.0. Arrows indicate the locations of bacteria or accumulation of either actin or pMLC.

To confirm this possibility, cell lysates were subjected to immunoblotting with anti-phospho-PAK1 antibody. We observed significant phosphorylation of PAK1 in Wt-PAK1/HBMEC at all time periods with no discernible decrease (Fig. 6A). Reprobing of the same blot with anti-PAK1 antibody showed the presence of equal quantities of protein in each lane. Correspondingly, the phosphorylation pattern of MLC showed no difference at any time period, although the level of phosphorylation of MLC was significantly lower compared to nontransfected HBMEC. The cAc-PAK1/HBMEC also showed high levels of phospho-PAK1 without observable difference at any time period (Fig. 6B). The levels of MLC phosphorylation

in these lysates were similar or less than those of Wt-PAK1/HBMEC despite the presence of reasonable quantities of MLC on the blot. In contrast, DN-PAK1/HBMEC showed a general decrease in PAK1 phosphorylation at all time points and significantly less PAK1 phosphorylation compared to the phosphorylation of PAK1 in nontransfected or pcDNA3-transfected HBMEC (Fig. 6C). The cell lysates of DN-PAK1/HBMEC showed an increase of MLC phosphorylation at 10 min and continued for up to 30 min. Interestingly, none of these transfected HBMEC showed detectable quantities of phospho-PAK2, and this could be due to presence of phospho-PAK1 in significant quantities in these cells. However, we

A

B

FIG. 4. Reduced phosphorylation of PAK1 at Thr-423 in *E. coli* invasion. (A) The total cell lysates of HBMEC infected with either E44 or E91 (50 μ g/lane) were separated on SDS-12% polyacrylamide gels and immunoblotted with anti-phospho-PAK1 antibody, followed by horseradish peroxidase-conjugated anti-rabbit immunoglobulin G. The blot was stripped and reprobed with anti-PAK1 antibody to show equality of loading. (B) The intensity of the upper bands was quantitated as described in Materials and Methods. The intensities were expressed as areas of the bands for each time period. The data represent the means $\pm$ the standard deviations from at least three separate experiments.

observed phospho-PAK2 protein in pcDNA3/HBMEC lysates by Western blotting with the antibody similar to that of nontransfected HBMEC (data not shown). These results clearly indicate that increased activity of PAK1 results in decreased phosphorylation of MLC, which could be due to inactivation of MLCK by activated PAK1. No increase in the phosphorylation of MLC either in Wt-PAK1- or cAc-PAK1-overexpressing HBMEC suggests that PAK1 may not be involved in direct phosphorylation of MLC as reported for other cell types (25).

Phospho-MLC association with the actin condensation site beneath the *E. coli* is blocked in Wt-PAK1 and cAc-PAK1 but not in DN-PAK1-transfected HBMEC. To further understand whether the inhibition of *E. coli* invasion into either Wt-PAK1- or cAc-PAK1-transfected HBMEC is due to the absence of actin condensation and/or absence of pMLC recruitment to the site of bacterial entry, immunocytochemistry analyses of transfected HBMECs were carried out as described above. As shown in Fig. 7, Wt-PAK1/HBMEC showed a change in basic morphology of HBMEC from a spindle or round shape to an extended leaf-like structure. The cells also showed leading edges similar to those of a migrating cell; however, the actin fibers did not differ significantly in number compared to those of either control nontransfected or pcDNA3-transfected HBMEC (similar to Fig. 3B). The Wt-PAK1/HBMEC infected with E44 showed several bacteria attached to the surface (Fig. 7E), with some of the bacteria having induced the condensation of actin at the site of entry. However, the actin accumulation was not as dense as that of the actin observed in normal HBMEC (Fig. 7F). The pMLC staining of these cells showed a punctate pattern distributed throughout the cell even after

infection with E44, and no association with the condensed actin was observed (Fig. 7H). However, cAc-PAK1/HBMEC exhibited slightly greater number of stress fibers compared to nontransfected cells (Fig. 8B). Similar to that of Wt-PAK1/HBMEC, these cells, when infected with E44, showed groups of bacteria attached to the surface and associated actin condensation beneath particular groups of bacteria (Fig. 8E and F). Again, the density of actin accumulation at the bacterial entry site was not similar to that observed in normal HBMEC. Also, no pMLC colocalization was observed at the bacterial entry site (Fig. 8G and H). Interestingly, overexpression of DN-

A

B

C

FIG. 5. Overexpression of Wt-PAK1 and cAc-PAK1 in HBMEC block *E. coli* invasion. (A) Diagram depicting the characteristics of various mutants of PAK1. (B) Total cell lysates (40 μ g of proteins) of HBMEC transfected with PAK1 constructs, along with nontransfected HBMEC, were separated on a SDS-12% polyacrylamide gel and then immunoblotted with anti-PAK1 antibody. The blot was stripped and reprobed with anti-Myc antibody. The arrow indicates the PAK1 band. (C) HBMEC transfected with different PAK1 constructs were infected with OmpA⁺ *E. coli* (E44), and the invasion assays were carried out as described in Materials and Methods. pcDNA3/HBMEC infected with OmpA⁻ *E. coli* (E91) was used as a control. The invasion of E44 was expressed as the percent invasion of E44 into pcDNA3/HBMEC being taken as 100% and are from at least three separate experiments performed in triplicates. The error bars indicate the standard deviation from the mean. The inhibition of E44 invasion is significant compared to control cells ($P < 0.01$ [unpaired two-tailed t test]). Similarly, the total cell-associated bacteria was represented as binding for each transfectant.

FIG. 6. Phosphorylation levels of PAK1 and MLC in Wt-, cAc-, and DN-PAK1-transfected HBMEC. Total cell lysates (50 μ g of proteins) of HBMEC transfected with either Wt-PAK1 (A), cAc-PAK1 (B), or DN-PAK1 (C) infected with E44 for various periods of time were separated on SDS-12% polyacrylamide gels and then immunoblotted with anti-phospho-PAK1 antibody. The bottom portions of the blots were probed with anti-pMLC-antibody. The blots were stripped and reprobed with either anti-PAK1 antibody or anti-MLC antibody to show the equal loading of proteins on to the gel.

PAK1 showed some change in the morphology of HBMEC, although not as prominent as that observed in Wt-PAK1 and cAc-PAK1-transfected HBMEC. However, no significant differences in stress fiber formation were observed (Fig. 8I and J). It is interesting that more pMLC staining was observed in the nucleus of these cells. These HBMEC, when infected with E44, showed significant accumulation of actin beneath some groups of bacteria, whereas other nearby bacteria showed no such accumulation (Fig. 8N). Anti-pMLC antibody staining revealed a strong association of pMLC beneath these groups of bacteria and colocalization of pMLC with actin (Fig. 8O and P). These results suggest that overexpression of Wt-PAK and cAc-PAK in HBMEC inhibited the *E. coli* invasion by inhibiting the phosphorylation of MLC necessary for its recruitment to the actin condensation sites and probably to generate the force to pull bacteria into the cell. These results also suggest that direct phosphorylation of MLC by PAK1, as shown in cell migration studies, is not operating in *E. coli* invasion.

Activation of FAK and PKC- α was not affected in HBMEC transfected with either Wt-PAK1 or cAc-PAK1. Previous studies from our laboratory showed that the invasive *E. coli* induces the phosphorylation of FAK and also PKC- α (22, 27). The activation of PKC- α has been shown to be downstream of FAK and is PI 3-kinase dependent. Dominant-negative forms of either FAK (FRNK) and PKC- α (PKC-CAT/KR) in HBMEC

significantly blocked the invasion of *E. coli* into these transfectants, suggesting the important role played by these molecules. Thus, to examine whether overexpression of either Wt-PAK or cAcPAK1, which showed an inhibitory effect on the invasion, affected other important events necessary for the invasion pro-

FIG. 7. Actin and pMLC patterns of HBMEC transfected with Wt-PAK1 by immunofluorescence. Transfected HBMEC were plated in eight-well chamber slides and grown to confluence. The monolayers were incubated with E44 for 15 min, washed, and fixed with 2% paraformaldehyde. The monolayers were then stained with either rhodamine-phalloidin (B and F) or anti-pMLC antibody (C and G). The monolayers were further incubated with Alexa-coupled secondary antibody to identify the anti-pMLC antibody and then mounted by using antifade solution containing DAPI. (A and E) The bacteria were visualized by using transmitted light optics with blue filter. (D and H) The cells were also visualized with dual filter mode for both red and green fluorescence. The confocal images were assembled and labeled by using Adobe Photoshop 6.0. Arrows indicate the locations of bacteria or accumulation of actin.

FIG. 8. Colocalization of actin and pMLC in DN-PAK1/HBMEC infected with E44. Both cAc-PAK1/HBMEC (A to H) and DN-PAK1/HBMEC (I to P) were infected with E44 for 15 min, fixed, and stained with rhodamine-phalloidin (B, F, J, and N) and anti-pMLC antibody (C, G, K, and O). The monolayers were further incubated with Alexa-coupled secondary antibody to localize the pMLC and mounted with antifade solution containing DAPI. (A, E, I, and M) The bacteria were visualized by using transmitted light optics with a blue filter. (D, H, L, and P) The cells were also visualized with dual filter mode for both red and green fluorescence. The confocal images were assembled and labeled by using Adobe Photoshop 6.0. Arrows indicate the locations of bacteria or accumulation of either actin or pMLC.

cess, FAK and PKC- α activation patterns were assessed. Equal quantities of proteins from the total cell lysates of Wt-PAK1/HBMEC infected either with E44 or E91 for various periods of time were subjected to immunoprecipitation with a polyclonal anti-FAK antibody. The immune complexes were then analyzed by immunoblotting with monoclonal anti-phosphotyrosine antibody (4G10). The phosphorylation of FAK increased between 10 and 15 min postinfection with E44, followed by a decline at 30 min. In contrast, E91 did not show

such an increase in the phosphorylation of FAK (Fig. 9A). In addition, the phosphorylation pattern of FAK in DN-PAK1/HBMEC lysates was similar to that of the pattern observed in pcDNA3/HBMEC infected with E44 (data not shown).

To assess the activation of PKC- α in these HBMEC transfectants, a nonradioactive PepTag assay was performed (27). As shown in Fig. 9B, no significant differences were observed in the PKC- α activation between Wt-PAK1/HBMEC and pcDNA3/HBMEC or DN-PAK1/HBMEC (only pcDNA3

FIG. 9. Activation of FAK and PKC- α in HBMEC overexpressing Wt-PAK1. (A) The monolayers of HBMEC transfected with Wt-PAK1 were incubated with either E44 or E91 for various periods of time, total cell lysates were prepared, and a fraction of the lysates (300 μ g of proteins) were immunoprecipitated with anti-FAK antibody (rabbit). The immunocomplexes were separated by SDS-10% polyacrylamide gels and Western blotted with anti-phosphotyrosine antibody (mouse). The blot was stripped and reprobed with anti-FAK antibody (rabbit). (B) Total cell lysates (20 μ g of proteins) of pcDNA3/HBMEC and Wt-PAK1/HBMEC infected with E44 for various periods of time were analyzed for PKC- α activity by a PepTag nonradioactive assay.

picture is shown). The activation of PKC- α increased up to 15 min postinfection, followed by a decline by 30 min in all of the cell lysates. E91, which was used as a negative control, showed no activation of PKC- α (data not shown). Both FAK phosphorylation and PKC- α activation were also assessed in cAc-PAK1/HBMEC and showed results similar to those observed in Wt-PAK1/HBMEC (similar to Fig. 9). Taken together, these results suggest that the inhibition *E. coli* invasion by overexpression of Wt-PAK1 or cAc-PAK1 in HBMEC is due to decreased MLC phosphorylation but not due to the blockade of other signaling pathways necessary for invasion.

DISCUSSION

Invasive *E. coli* that causes meningitis interact with the BMEC that form the lining of the blood-brain barrier in order to invade the central nervous system. The bacterium manipulates the host cell cytoskeleton, which is primarily modulated by actin and myosin, for the invasion. Our studies showed that the OmpA of *E. coli* interacts with a 95-kDa HBMEC receptor to invade HBMEC (18). This event induces the phosphorylation of FAK, PI 3-kinase, and PKC- α , which have been shown to promote the actin reorganization required for *E. coli* invasion of HBMEC (22, 23, 27); however, the role of myosin, another cytoskeletal component, in *E. coli* invasion of HBMEC has yet to be addressed. Data presented in the present study reveal two important findings. First, invasive *E. coli* induces the phosphorylation of the regulatory light chain (MLC) of myosin II at Ser-19. The phosphorylated MLC is recruited to the actin accumulation points at the bacterial attachment site. Second, PAK1 activation was downregulated by *E. coli*, which probably resulted in MLCK inactivation required for more MLC phosphorylation.

The extent of MLC phosphorylation represents a balance

between the relative activities of MLCK and myosin phosphatase, both of which are subject to extensive regulation (10). However, PAK1, the upstream regulator of MLCK has also been shown to directly phosphorylate MLC in fibroblasts (25). The results of ML-7 inhibition of *E. coli* invasion, however, indicate that MLCK may be playing a major role in *E. coli*-induced phosphorylation of MLC. In addition, PAK1 has no measurable effect on MLC phosphorylation when either Wt-PAK1 or cAc-PAK1 overexpressed in HBMEC, suggesting that PAK1 may not directly phosphorylate MLC in the *E. coli* invasion process. Alternatively, PAK1 might induce MLC phosphorylation indirectly, via activation of RhoA-kinase (ROCK), which also affects MLCK activity (29). The ROCK pathway leads to stress fiber formation and focal adhesion formation by inhibiting myosin phosphatase activity and thereby enhancing MLC phosphorylation. Interestingly, ROCK may directly phosphorylate MLC in stress fibers, whereas MLCK phosphorylates MLC in cortical bundles (7, 9, 29). However, inhibition of ROCK by HA177 (100 nM, an inhibitor of ROCK) showed no effect on *E. coli* invasion and phosphorylation of MLC (unpublished results), suggesting the minimal role of ROCK in MLC phosphorylation. Since differential roles are played by ROCK and MLCK in spatial regulation of MLC phosphorylation (29), it is possible that other signaling events might also be involved (i.e., ROCK activated MLC phosphorylation), although to a lesser extent, depends on how *E. coli* invades the HBMEC (from center or periphery). Besides PAK1, PKC- α has also been suggested to induce phosphorylation of MLC (14, 15). Since Wt-PAK1 and cAc-PAK1 overexpression in HBMEC did not alter the PKC- α activity, as estimated by a PepTag assay, it is possible that PKC- α does not have a role in inducing the phosphorylation of MLC. Despite the exact roles of various molecules in phosphorylating MLC, the recruitment of pMLC to the site of bacterial entry clearly indicates a role for myosin in the invasion process, and for the first time we report here this observation in microbial pathogenesis.

Our studies further show the potential role of PAK1, a putative effector of either Rac1 or Cdc42, in *E. coli* invasion of HBMEC (Fig. 10). Infection of HBMEC with invasive *E. coli*

FIG. 10. Schematic representation of *E. coli*-induced phosphorylation of MLC. The scheme shows the interaction of Rac1/Cdc42 with PAK1 for subsequent effect on MLC phosphorylation. (i) *E. coli* and DN-PAK1 inhibit this interaction, which results in increased activity of MLCK and increased phosphorylation of MLC. The phosphorylated MLC is recruited to actin accumulation sites for the invasion of *E. coli*. (ii) Overexpression of either Wt-Pak1 or cAc-PAK1 in HBMEC reduces the MLCK activity with a concomitant decrease in MLC phosphorylation; thus, there is no invasion of *E. coli*.

resulted in a significant reduction in the phosphorylation of PAK1 at Thr-423. The downregulation of PAK1 activity was rapid and short lived, with reduced phosphorylation between 10 and 15 min, followed by a return of normal levels thereafter. A corresponding increase in MLC phosphorylation suggests that decreased kinase activity of PAK1 resulted in increased MLCK activity. Consistent with this concept, overexpression of cAc-PAK1, which had enhanced kinase activity, blocked the *E. coli* invasion of HBMEC, whereas the DN-PAK1 mutant, which lacks kinase activity, showed no effect. Overexpression of Wt-PAK1 also blocked the invasion of *E. coli* probably due to enhanced basal level activity of PAK1. Since these constructs did not block the formation of stress fibers in HBMEC, the observed effect are unlikely to be due to nonspecific responses. These results are in sharp contrast to those observed in endothelial cell migration studies, in which microinjection of DN-PAK1 or cAc-PAK1 significantly inhibited cell migration (11). Other reports in various systems have been similar to our studies, as an active PAK1 decreased the phosphorylation of MLC in BHK-21 and HeLa cells (24). Thus, the effects of PAK1 appear to be cell type dependent and possibly account for these apparent discrepancies.

Chen et al. have shown that PAK1 activation is important for *Salmonella*-induced nuclear response but not for actin-mediated entry into cultured cells (2). These responses are dependent on the interaction of Cdc42. However, a dominant-negative Cdc42 mutant did not block invasion of *Salmonella*, thereby indicating a role for Rac1. These studies further showed that Rac1 was activated during the *Salmonella* entry, which was blocked by the overexpression of a dominant inhibitory form of Rac1. The role of Rac1/Cdc42 in modulating the MLC phosphorylation and subsequently in *E. coli* invasion is currently being investigated.

Actin rearrangements are important for *E. coli* invasion of HBMEC (21). Our previous studies utilizing dominant-negative FAK or PKC- α mutants showed that they prevented actin accumulation at the bacterial entry site, thus blocking the invasion. In contrast, overexpression of either Wt- or cAc-PAK1 that blocks *E. coli* invasion still allowed for actin condensation, although this was not as dense as that of control cells. The significant difference between control and PAK1 overexpressed HBMEC is the lack of pMLC at the bacterial entry site, indicating the requirement of myosin-driven motor function for the internalization of the bacteria. Although we have shown that OmpA interaction with HBMEC receptor is important for the actin condensation, it is still remain to be elucidated how the invading *E. coli* cause the downregulation of PAK1 activity. In several other bacterial invasion mechanisms, the effector molecules are carried out by a specialized protein secretion system and translocation apparatus type III. SopE, a serovar Typhimurium secreted protein, was characterized as a GEF for Rho-GTPases by its ability to stimulate in vitro nucleotide exchange on Cdc42, Rac1, and RhoA and is required for entry (2). However, no secretion system has been identified in *E. coli* thus far. Nonetheless, OmpA interaction appears to be crucial for the further expression of other virulence genes, whose products (secreted or surface expressed) may modulate the function of PAK1 either directly or indirectly. Our present view is that two different signaling pathways operate in HBMEC upon contact with *E. coli*, one actin rear-

angement related (FAK, PI 3-kinase, and PKC- α) and another myosin related (Rac1/Cdc42, PAK1, and MLC), whose convergence is required at the *E. coli* entry site for efficient internalization of bacteria. A possible mechanism of MLC phosphorylation via the Rac1/Cdc42 and PAK1 pathway involved in *E. coli* invasion of HBMEC is shown in Fig. 10. In this model, both the *E. coli* and the DN-PAK1 inhibit the interaction of Rac/Cdc42 with PAK1, thus increasing the MLCK activity with a subsequent increase in MLC phosphorylation. The pMLC recruits to the actin accumulation sites beneath the bacteria to pull the *E. coli* into the HBMEC (left side of the Fig. 10). In contrast, overexpression of cAc-PAK1 decreases the MLCK activity, which in turn reduces the MLC phosphorylation. Thus, no pMLC is available for recruitment to the site *E. coli* entry, resulting in no invasion (right side of Fig. 10). Overexpression of Wt-PAK1, on the other hand, interacts with the total cellular Rac1/Cdc42 that is present in a significantly more active form, thus reducing the activity of MLCK. Although *E. coli* could block the Rac/Cdc42 interaction with Wt-PAK1 in these cells to a certain extent, the basal level activity of PAK1 is significantly high to block the *E. coli* invasion.

Our results, in summary, strongly support a role for MLC phosphorylation in the *E. coli* invasion of HBMEC. The recruitment of pMLC, along with actin, beneath the bacteria is crucial for invasion. Importantly, *E. coli*-induced MLC phosphorylation is regulated by PAK1. As far as we are aware, this is the first time that the role of myosin has been investigated in detail for any bacterial pathogen.

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