

Listeria monocytogenes, an Invasive Bacterium, Stimulates MAP Kinase upon Attachment to Epithelial Cells

Patrick Tang, Ilan Rosenshine, and B. Brett Finlay

Biotechnology Laboratory and the Departments of Biochemistry and Microbiology,
University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada

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Protein tyrosine phosphorylation is an important regulatory mechanism for many cellular processes in eucaryotic cells. During the invasion of the gram-positive pathogen, *Listeria monocytogenes*, into host epithelial cells, two host proteins become tyrosine phosphorylated. We have identified these major tyrosine phosphorylated species to be two isoforms of mitogen-activated protein (MAP) kinase, the 42 and 44 kDa MAP kinases. This activation begins within 5 to 15 min of bacterial infection. The tyrosine kinase inhibitor, genistein, blocks invasion as well as the tyrosine phosphorylation of these MAP kinases. Using cytochalasin D to block bacterial internalization but not adhesion, we showed that bacterial adherence rather than uptake is required for MAP kinase activation. Internalin mutants, which are unable to adhere efficiently to host cells, do not trigger MAP kinase activation. Other invasive bacteria, including enteropathogenic *Escherichia coli* (EPEC), and *E. coli* expressing *Yersinia enterocolitica* invasin, were not observed to activate MAP kinase during invasion into cultured epithelial cells. These results suggest that *L. monocytogenes* activates MAP kinase during invasion and a MAP kinase signal transduction pathway may be involved in mediating bacterial uptake.

INTRODUCTION

The gram-positive bacterium, *Listeria monocytogenes*, is a facultative intracellular parasite capable of causing severe disease in immunocompromised humans and animals (Seeliger, 1961; Gray and Killinger, 1966). *L. monocytogenes* is ubiquitous in nature, occurring in its natural soil environment in all regions of the world (Ryser and Marth, 1991). Outbreaks of listeriosis have been correlated with the consumption of *Listeria*-contaminated foodstuff, especially dairy products (Schlech *et al.*, 1983; Fleming *et al.*, 1985; Linnan *et al.*, 1988; Farber and Peterkin, 1991). Listeriosis usually presents as meningitis, bacteremia, or prenatal infections.

The murine model of listeriosis is well documented because *L. monocytogenes* has been used extensively as a pathogen to study T cell-mediated immunity (Mackness, 1962; Hahn and Kaufmann, 1981). However, a molecular understanding of *L. monocytogenes* pathogenicity has only recently begun to emerge. Using tissue culture models of infection, several virulence factors of

L. monocytogenes have been characterized (reviewed in Portnoy *et al.*, 1992). A global regulator, PrfA, controls the transcription of virulence-related genes including *inlA*, *hly*, *actA*, and *plcB* (Mengaud *et al.*, 1991; Chakraborty *et al.*, 1992; Leimeister-Wachter *et al.*, 1992; Dramsi *et al.*, 1993). PrfA is a transcription factor that is regulated by bacterial growth phase and temperature (Mengaud *et al.*, 1991; Leimeister-Wachter *et al.*, 1990, 1992; Freitag *et al.*, 1993). Internalin encoded by *inlA* is required for invasion into the host cell (Gaillard *et al.*, 1991). Transposon mutants in *inlA* have a highly reduced invasion efficiency into a variety of cultured cell lines. The closely related noninvasive *Listeria innocua* is rendered invasive when the *inlA* gene from *L. monocytogenes* is expressed in *L. innocua*. The transformed *L. innocua* do not invade as efficiently as wild type *L. monocytogenes*, which suggests that other unidentified bacterial factors also take part in the invasion process (Gaillard *et al.*, 1991). At present, internalin is the only factor shown to be involved in the initial entry process of *Listeria* infection.

Listeriolysin O encoded by *hly* allows the bacterium to escape from the phagosome into the cytoplasm of the host cell (Portnoy *et al.*, 1988). Bacteria in the cytoplasm use ActA to polymerize host actin filaments, enabling them to move intracellularly (Tilney and Portnoy, 1989; Kocks *et al.*, 1992; Theriot *et al.*, 1992; Neibuhr *et al.*, 1989). Some bacteria are extruded from the cell in pseudopod-like structures and phagocytosed by adjacent cells, allowing the bacteria to spread from cell to cell while remaining intracellular (Gaillard *et al.*, 1987; Tilney and Portnoy, 1989; Vazquez-Boland *et al.*, 1992). While much effort has gone into the study of the intracellular activities of *L. monocytogenes*, little is known about the initial internalization process.

Invasive bacteria appear to send signals to the host cell to trigger their internalization. *Salmonella typhimurium*, enteropathogenic *Escherichia coli* (EPEC), and *Yersinia pseudotuberculosis* have been shown to take advantage of host signaling mechanisms to gain entry into the cell (reviewed in Bliska and Falkow, 1993). These bacteria alter tyrosine phosphorylation in the host cell during their invasion. Galan and coworkers have demonstrated tyrosine phosphorylation of the epidermal growth factor receptor (EGFR) during the invasion of *S. typhimurium* into Henle-407 (Galan *et al.*, 1992). The addition of exogenous EGF was sufficient to induce internalization of noninvasive *invA* mutants. However, EGFR may only be one of several mechanisms for *S. typhimurium* invasion as protein tyrosine kinase inhibitors do not block *S. typhimurium* entry into HeLa cells (Rosenshine *et al.*, 1992b). Tyrosine phosphorylation of a 90-kDa cytoskeletal protein, Hp90, in EPEC infected cells was observed by Rosenshine and colleagues (1992a). Hp90 is involved in the assembly of the cytoskeletal structure under the attached bacterium and in the initialization of the uptake process. Tyrosine kinase inhibitors block Hp90 phosphorylation as well as invasion of EPEC. These inhibitors also block the invasion of *E. coli* expressing *Y. enterocolitica* invasins into epithelial cells (Rosenshine *et al.*, 1992b).

In this work, we examined the signals transmitted in the host epithelial cell during the uptake of *L. monocytogenes*. We have identified host proteins that are tyrosine phosphorylated during the internalization process.

MATERIALS AND METHODS

Bacterial Strains

The *L. monocytogenes* strain 1/2a₃ is a streptomycin resistant derivative of SLCC 5764. Strain 4b₁ is a streptomycin derivative of ATCC 13932. M44 is a noninvasive Tn916ΔE mutant of 4b₁. These strains were kindly provided by S. Kathariou from the University of Hawaii. The *L. monocytogenes* strain EGD SmR (bof297) is the parent of BUG 11, a noninvasive mutant with a Tn1545 insertion upstream of *inlA* and *inlB* (Gaillard *et al.*, 1991). Both the parent and mutant were provided by P. Cossart from Institut Pasteur. All *Listeria* were grown in brain heart infusion broth with shaking at 37°C until logarithmic phase

(0.5 to 0.8 O.D.₆₀₀). Antibiotics, when appropriate, were added at the following concentrations: streptomycin 600 μg/mL, spectinomycin 60 μg/mL, and erythromycin 10 μg/mL (Sigma, St. Louis, MO).

E. coli expressing *Y. enterocolitica inv* (HB101/pVM101) and EPEC E2348/69 were grown to stationary phase in Luria-Bertrani broth at 37°C without shaking. All bacteria growth media were obtained from Difco (Detroit, MI).

Tissue Culture Cells

HeLa, Henle-407 and CaCo-2 cells were cultured in Eagle's minimum essential medium (MEM) (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (GIBCO) and penicillin (100 U/mL)/streptomycin (50 μg/mL) (GIBCO). Cells were kept at 37°C with 5% CO₂ and used between passages 10 and 40.

Invasion Assay

The number of internalized bacteria was quantitated using the invasion assay in 24-well tissue culture plates. Tissue culture cells were washed once with PBS and then incubated with antibiotic-free MEM for at least 60 min prior to infection. When appropriate, inhibitors were added 30 min prior to infection with bacteria. Bacteria were washed once with PBS and then resuspended in MEM before being added to the monolayer at a multiplicity of infection between 10 and 100. After allowing the specified time for invasion (1 h for *L. monocytogenes* and pVM101/HB101, and 3 h for EPEC unless otherwise specified), the monolayer was washed with phosphate-buffered saline (PBS). The cells were then incubated for 90 to 120 min with MEM containing 100 μg/mL gentamicin (Sigma) to kill extracellular bacteria. The monolayer was again washed with PBS. Triton X-100 (1%) was added to lyse the cells, releasing intracellular bacteria. These bacteria were diluted and quantitated by plating onto trypticase soy agar plates or LB agar plates with antibiotics when appropriate.

Preparation of Cell Lysates

Cells were grown to confluency on 100-mm tissue culture plates (Costar, Cambridge, MA) and serum-starved prior to use. After appropriate treatment, the cell monolayer was washed with ice-cold PBS and removed with a cell scraper (Costar). The cells were concentrated by briefly pelleting and removing the supernatant. A lysis buffer (1% Triton X-100, 50 mM tris(hydroxymethyl)aminomethane [Tris]-HCl, pH 7.5, 0.4 mM NaVO₄, 1 mM NaF, 0.1 mg/mL phenylmethylsulfonyl fluoride [PMSF], and 10 μg/mL leupeptin) was added to break open cells. The Triton X-100 insoluble fraction consisting of cytoskeletal and nuclear components as well as bacterial proteins was collected by centrifugation at 16000 × g. The soluble fraction contained the cytosol and membrane. The entire process was carried out at 4°C to minimize protein degradation. Protein concentration was determined by using the bicinchoninic acid assay (Sigma).

SDS-PAGE and Western Immunoblot Analysis

Samples were prepared for electrophoresis by mixing with concentrated sodium dodecyl sulfate (SDS) reducing buffer to a final concentration of 100 mM Tris-HCl, pH 6.8, 2% SDS, 0.1 M dithiothreitol (DTT), and 10% glycerol and boiling for 7 min. These samples were then separated on polyacrylamide gels according to the methods of Laemmli (1970). Proteins were electrophoretically transferred to nitrocellulose (AB-S 83, Schleicher and Schuell, Keene, NH) using the Pharmacia LKB Multiphor II system (Uppsala, Sweden). The membrane was blocked with 4% bovine serum albumin (BSA) in TBS (150 mM NaCl and 20 mM Tris-HCl, pH 7.5) for overnight at 4°C. The membrane was washed once with Tris-buffered saline (TBS) before the addition of primary antibodies that were diluted in TBS with 1% BSA. The anti-phosphotyrosine monoclonal, 4G10, was purchased from UBI (Lake Placid, NY). The anti-mitogen-activated protein (MAP) kinase and related antibodies: anti-erk1-III (R1), anti-erk1-CT (R2),

anti-erk1-NT (R3) and anti-p44^{mpk}, and anti-cdc2-CT were gifts from S. Pelech (Kinetek Biotechnology, Vancouver, BC, Canada). After overnight incubation at 4°C, the blots were washed twice with TBS for 5 min, twice with TBS containing 0.05% NP-40 for 10 min, and again in TBS for 10 min before adding the secondary antibodies (goat anti-mouse or goat anti-rabbit coupled to alkaline phosphatase). The blots were incubated for 1 h at room temperature with gentle shaking and then washed as before. The membrane was washed once in AP substrate buffer (100 mM NaCl, 100 mM Tris-HCl, pH 9.5, 5 mM MgCl₂) for 10 min before color development with 0.5 mg/mL 5-bromo-4-chloro-3-indolyl phosphate (GIBCO) and 0.25 mg/mL nitro blue tetrazolium (GIBCO) in AP substrate buffer. The reaction was stopped after 5 to 30 min by washing in a large volume of water.

Immunoprecipitation

Cell lysates were added to 4G10-conjugated agarose in Buffer A (50 mM Tris-HCl, pH 8.0, 10% glycerol, 138 mM NaCl, 1% NP-40, 0.4 mM NaVO₄, and 100 µg/mL PMSF) and gently rotated at 4°C inside microfuge tubes overnight. The immunoprecipitate was then washed twice with Buffer B (150 mM NaCl, 0.2% NP-40, 2 mM EDTA, and 10 mM Tris-HCl, pH 7.5) and once with Buffer C (10 mM Tris-HCl, pH 7.5). Then 2.5× SDS reducing buffer was added and the sample was boiled for 7 min to release immunoprecipitated proteins. The supernatant fraction was resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and Western immunoblotting as described above.

Anion Exchange Chromatography

Cell extracts (5 mg protein) were loaded onto a 1 mL Econo-Pac Q anion exchange column (Bio-Rad, Richmond, CA) in Buffer D (5 mM 3-(*N*-morpholino)propanesulfonic acid [MOPS], pH 7.2, 5 mM ethylene glycol-bis(β -aminoethyl ether) [EGTA], 1 mM NaVO₄, and 1 mM DTT) at a flow rate of 1 mL/min in a Bio-Rad Econo System. The column was developed with a 10 mL linear gradient of 0 to 0.8 M NaCl in Buffer D. The column fractions (300 µL) were assayed for phosphorylating activity towards myelin basic protein (MBP). Five microliters of sample were diluted to a total volume of 25 µL with 1 mg/mL MBP, 0.5 µM cyclic 3',5'-cyclic monophosphate (cAMP) dependent protein kinase inhibitor peptide, 50 µM [γ -³²P]ATP (1800 cpm/pmol) (Amersham, Arlington Heights, IL), and assay dilution buffer (30 mM β -glycerol phosphate, 20 mM MOPS, pH 7.2, 20 mM MgCl₂, 5 mM EGTA, 1 mM DTT, and 0.5 mM NaVO₄). The kinase reaction was started with the addition of [γ -³²P]ATP and allowed to continue for 5 min at 30°C. The reaction was terminated by spotting 20 µL of each sample onto Whatman P81 phosphocellulose paper (Maidstone, England). The filter papers were washed 10 to 15 times in 1% phosphoric acid and then transferred to scintillation vials. The degree of MBP phosphorylation was determined by adding scintillation fluid and counting in a scintillation counter.

RESULTS

L. monocytogenes Causes Host Protein Tyrosine Phosphorylation During Invasion

Tyrosine phosphorylation is crucial in the regulation of many eucaryotic processes (Glenny, 1992). Recent work has revealed that bacterial pathogens such as *S. typhimurium*, *Y. pseudotuberculosis*, and EPEC are capable of modifying host tyrosine phosphorylation (Bliska and Falkow, 1993). We examined the role of tyrosine phosphorylation in the uptake of invasive bacteria including *L. monocytogenes*, EPEC, and *E. coli* expressing *Y. enterocolitica* invasin (HB101/pVVM101). Using anti-phosphotyrosine Western immunoblotting, a tyrosine phosphorylated protein was detected in Henle-407 cells

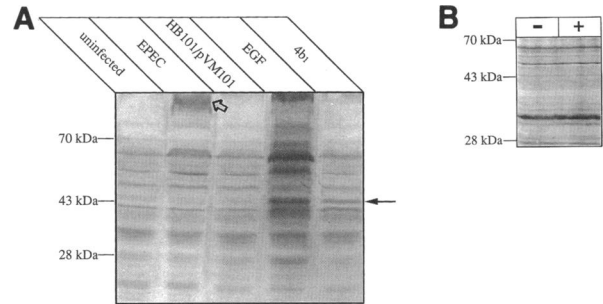


Figure 1. Western immunoblot of tyrosine phosphorylated proteins after various treatments. (A) Henle-407 cells were left untreated (uninfected), treated with EGF (100 ng/mL) for 10 min, or infected with EPEC for 3 h, HB101/pVVM101 for 1 h, or *L. monocytogenes* (4b₁) for 1 h. The cell lysates were solubilized with Triton X-100 and immunoblotted with the anti-phosphotyrosine monoclonal antibody (4G10). The solid arrow on the right shows the position of the 44 kDa tyrosine phosphorylated protein induced by *L. monocytogenes* infection. The open arrow marks to the Hp90 tyrosine phosphorylated protein induced by EPEC infection. (B) 4G10 immunoblot of the Triton-insoluble fraction from HeLa cells uninfected (-) or infected with *L. monocytogenes* (1/2a₃) for 1 h (+). The positions of molecular weight standards are shown on the left of both blots.

infected with *L. monocytogenes* (Figure 1A). This Triton X-100 soluble protein has a molecular weight of ~44 kDa. No differences in the Triton X-100 insoluble fraction were observed for infected and uninfected cell lysates suggesting that protein translocation is not the reason for the observed increase of the tyrosine phosphorylated protein (Figure 1B).

Henle cells infected with EPEC showed induction of the Hp90 protein at 3 h post-infection, but no other proteins showed a significant increase in tyrosine phosphorylation (Figure 1A). Hp90 is a tyrosine phosphorylated protein that is involved in EPEC invasion (Rosenshine *et al.*, 1992a). This host protein becomes tyrosine phosphorylated and aggregates under the adherent EPEC. HB101/pVVM101 also did not induce a detectable increase in tyrosine phosphorylation in host cells after 1 h infection. However, tyrosine kinase inhibitors do block HB101/pVVM101 invasion (Rosenshine *et al.*, 1992b). Epidermal growth factor (EGF) treatment for 10 min caused a dramatic increase in tyrosine phosphorylation of many different proteins. The effect of EGF was much stronger than the phosphorylation induced by *L. monocytogenes* infection.

Similar results were observed in CaCo-2 cells (not shown) and HeLa cells infected with *L. monocytogenes* (Figure 2A). The induction of tyrosine phosphorylation was rapid and could be detected within 5 to 15 min after infection with *L. monocytogenes* (Figure 2A). The level of tyrosine phosphorylation reached a maximum at 60 min. The rate of bacterial invasion also levels off at this time (Figure 2B).

When extracellular bacteria were washed away at 30 min post-infection, the tyrosine phosphorylation of the

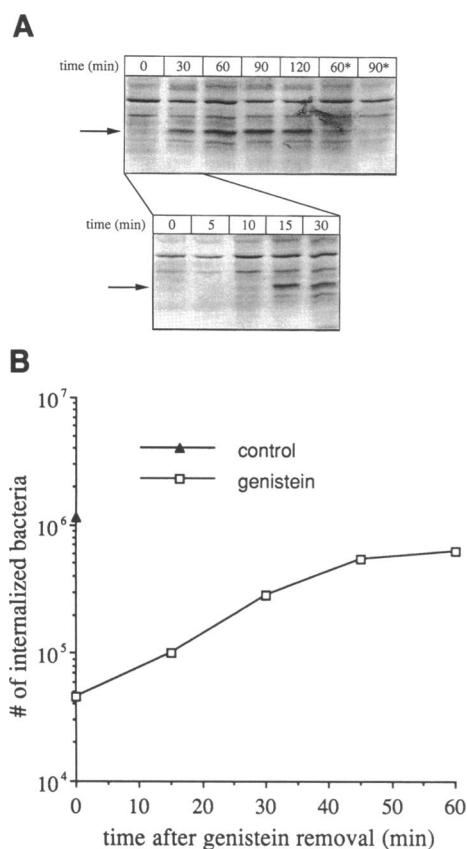


Figure 2. Correlation between the induction of the tyrosine phosphorylation and invasion. (A) Anti-phosphotyrosine immunoblot of lysates from HeLa cells infected with *L. monocytogenes* (1/2a₃) for various times. The asterisked time points (60* and 90*) indicate that the monolayer was washed twice with PBS to remove extracellular bacteria at 30 min post-infection. The monolayer was then incubated in fresh MEM for an additional 30 min (60*) or 60 min (90*). The arrow points to the position of the 44 kDa tyrosine phosphorylated protein induced by *L. monocytogenes* infection. (B) Kinetics of *L. monocytogenes* (1/2a₃) invasion of HeLa cells. The HeLa cell monolayer was infected with *L. monocytogenes*. Gentamicin (100 μg/mL) was added after various times to kill extracellular bacteria. The surviving internalized bacteria were quantitated by plating.

44 kDa protein was significantly reduced at 60 min post-infection (Figure 2A). This suggests that bacteria in the process of invasion, rather than already intracellular bacteria, are triggering the tyrosine phosphorylation of the 44 kDa protein. As well, the tyrosine phosphorylation of the 44 kDa protein is transient, being turned on or off by the presence or absence of invading bacteria.

The Tyrosine Kinase Inhibitor, Genistein, Blocks Invasion of *L. monocytogenes* as well as Induction of the Tyrosine Phosphorylated Protein

The tyrosine kinase inhibitor genistein was able to block the invasion of *L. monocytogenes* (Figure 3A). There was greater than a 20-fold decrease in invasion when host

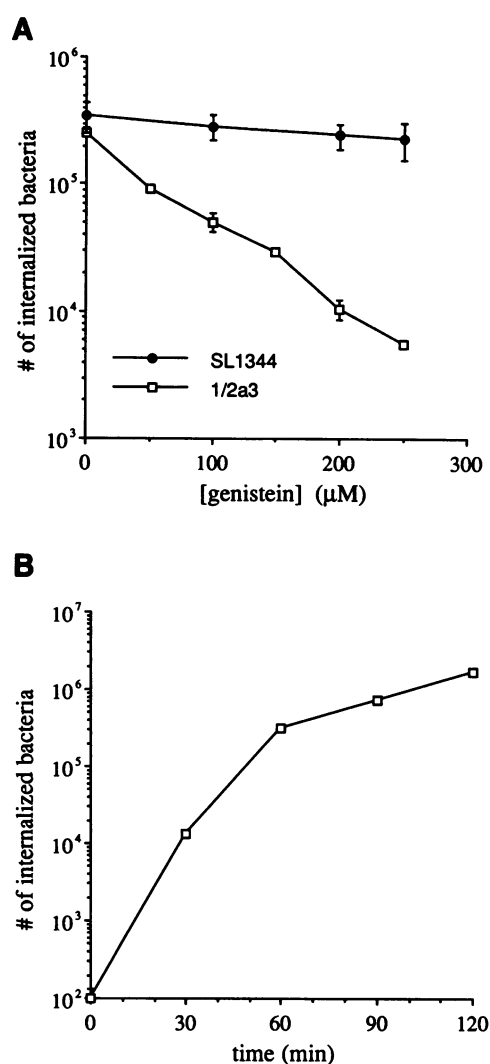


Figure 3. Effect of genistein treatment on bacterial invasion. (A) HeLa cells were pretreated with various concentrations of genistein 30 min prior to infection. The genistein was dissolved in equal volumes of dimethyl sulfoxide (DMSO) and added to the tissue culture media. *S. typhimurium* (SL1344) invasion was not significantly affected while *L. monocytogenes* (1/2a₃) uptake by HeLa cells was increasingly reduced with genistein concentration. (B) The genistein effect on *L. monocytogenes* invasion into HeLa cells was reversible. HeLa cells were pretreated with 200 μM genistein and infected with *L. monocytogenes* (1/2a₃) for 1 h. The genistein was washed away and intracellular bacteria were quantitated after various times as described before. The control represents the number of intracellular bacteria at 1 h post-infection without genistein treatment.

cells were treated with 200 μM genistein. At the same concentrations, *S. typhimurium* invasion was not significantly reduced. This was also observed by Rosen-shine *et al.* (1992b). In control experiments, genistein did not affect bacteria or host cell survival at the concentrations used. The effect of genistein was also highly reversible (Akiyama *et al.*, 1987; Hill *et al.*, 1990). Upon removal of the drug, bacterial invasion was restored to

normal untreated levels within an hour, demonstrating that genistein specifically interfered with the internalization process rather than adherence (Figure 3B). This phenomenon was also observed for EPEC and HB101/pVM101 (Rosenshine *et al.*, 1992a,b). In similar experiments, we found that the class of tyrosine kinase inhibitors known as tyrphostins also reduced invasion of *L. monocytogenes* into host cells.

Genistein also inhibited the bacterial-induced tyrosine phosphorylation of the 44 kDa protein (Figure 4). When HeLa cells were pretreated with genistein, infection with *L. monocytogenes* did not induce a significant level of tyrosine phosphorylation of the 44 kDa protein. Increased phosphorylation is still evident though it is highly reduced by genistein. It is not known whether genistein blocked the tyrosine phosphorylation of the 44 kDa protein itself or blocked the activation of a component upstream in the same pathway. This suggests that tyrosine phosphorylation of the 44 kDa protein is associated with invasion of *L. monocytogenes*.

The 44-kDa Protein Is MAP Kinase

Western immunoblots with several different antibodies showed that the induced protein has the same electrophoretic mobility as p44 MAP kinase (Figure 5A). Anti-erk1-III (R-1) and anti-erk1-CT (R-2) polyclonal antibodies detect the p44 MAP kinase isoform quite efficiently. Directly below the p44 MAP kinase band is the band representing the 42 kDa isoform of MAP kinase (p42 MAP kinase). MAP kinase must be phosphorylated on a threonine and a tyrosine residue for maximal activity. Increased tyrosine phosphorylation of MAP kinase is thus one indication of increased MAP kinase activity in the cell. Of particular interest are antibodies against seastar MAP kinase (p44^{mpk}), which have a higher affinity for the tyrosine phosphorylated forms of human MAP kinase than the inactive, unphosphorylated form (Posada *et al.*, 1991; Weinstein *et*

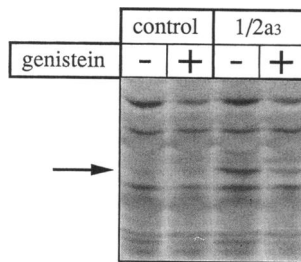


Figure 4. Anti-phosphotyrosine immunoblot of HeLa cells pre-incubated with 200 μ M genistein (+) or with an equal volume of its solvent, DMSO, (-) for 30 min. Cells were left uninfected (control) or were infected with *L. monocytogenes* (1/2a₃) for 1 h. The arrow marks the position of the 44 kDa tyrosine phosphorylated protein induced by *L. monocytogenes* infection. Genistein pretreatment reduced phosphorylation of the 44 kDa protein in infected HeLa cells. Internalization of *L. monocytogenes* was also reduced (Figure 3).

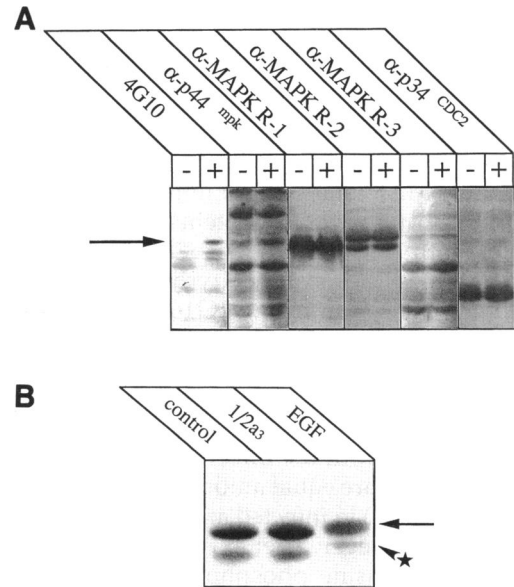


Figure 5. Western immunoblots probed with anti-phosphotyrosine, anti-MAP kinase and related antibodies. (A) HeLa cells were untreated (-) or infected with *L. monocytogenes* 1/2a₃ for 1 h (+). Cell lysates were immunoblotted with anti-phosphotyrosine antibodies (4G10), antibodies against sea-star p44^{mpk}, antibodies against various regions of rat p44^{erk1} (α -MAPK R-1, R-2, and R-3), and anti-cdc2 antibodies (α -p34^{cdc2}). The arrow shows the location of the 44 kDa tyrosine phosphorylated protein which co-migrates with the p44 MAP kinase band detected by the various anti-MAP kinase antibodies. Note that α -p44^{mpk} has a higher affinity for the 44 kDa protein in *L. monocytogenes* infected cells compared with uninfected cells. This antibody has a higher affinity for the tyrosine phosphorylated form of human MAP kinase. (B) α -MAPK R-2 immunoblot of HeLa cell lysates. HeLa cells were untreated (control), infected with *L. monocytogenes* (1/2a₃) for 1 h, or treated with EGF (100 ng/mL) for 10 min. The lanes were loaded with enough protein to allow visualization of the p42 MAP kinase band. The arrow points to the shifted (phosphorylated) form of p44 MAP kinase. The star (*) shows the position of the phosphorylated form of p42 MAP kinase.

et al., 1992). This antibody has a higher affinity for the p44 MAP kinase in infected HeLa cells than in uninfected cells demonstrating that the MAP kinase in *L. monocytogenes* infected cells are more highly tyrosine phosphorylated. Using the different anti-MAP kinase antibodies, we could not detect a change in the mobility of the MAP kinase bands between infected and uninfected cells. This suggests the MAP kinase activation was not as strong as that observed during growth factor stimulation (i.e., EGF) where obvious shifts in the mobility of the MAP kinase bands can occur due to the phosphorylation of the protein (Figure 5B). EGF stimulation induced tyrosine phosphorylation of both the p42 and p44 isoforms of MAP kinase, causing mobility shifts in almost the entire population of these MAP kinase species. In contrast, our inability to detect a shifted MAP kinase band in the *L. monocytogenes* infected cells suggests that only a small fraction of the MAP kinase is being tyrosine phosphorylated in response to bacterial

invasion (Figure 5A,B). A conservative estimate would place this amount to be less than 5% of the total MAP kinase in the cells based on the absence of a detectable band.

Immunoprecipitation with anti-phosphotyrosine (4G10)-conjugated agarose confirmed that the induced protein was MAP kinase. After immunoprecipitating the tyrosine phosphorylated proteins, Western immunoblotting with anti-MAP kinase antibodies was conducted to measure levels of tyrosine phosphorylated MAP kinase. As seen in the 4G10 immunoblots, there is a 44 kDa tyrosine phosphorylated band that induced by infection with both strains of *L. monocytogenes* (Figure 6A). This phosphoprotein can be immunoprecipitated by the 4G10-agarose beads, which pick up more of the 44-kDa protein from the infected cells. In the MAP kinase blots, there are equal amounts of MAP kinase (p42 and p44 isoforms) in infected and uninfected cell lysates (Figure 6B). However, after 4G10 immunoprecipitation to select for tyrosine phosphorylated proteins, the *L. monocytogenes* infected samples show more MAP kinase than the uninfected samples. This clearly shows that *L. monocytogenes* infection causes tyrosine phosphorylation of MAP kinase. A smaller isoform of MAP kinase, the p42 MAP kinase, was also shown to be tyrosine phosphorylated in infected cells (Figure 6B). This isoform was not as strongly activated as the p44 MAP kinase and thus was not readily detected in previous 4G10 immunoblots.

While tyrosine phosphorylation of MAP kinase is highly indicative of its activation, it is also necessary to measure its kinase activity. Cell lysates were fractionated by anion exchange chromatography and the fractions analyzed for myelin basic protein (MBP) phosphorylation activity, a method used to measure *in vitro* MAP kinase activity. Fractions containing MAP kinase were detected through Western immunoblotting with 4G10 and anti-erk1-CT (R-2) antibodies. Using the fractions containing MAP kinase, we found more MBP phosphorylation in the infected samples than in uninfected samples (Figure 7A). There was a 62% increase in the peak level of MAP kinase activity due to infection with *L. monocytogenes*. The amount of MAP kinase was equal in uninfected and infected samples as detected by immunoblotting with anti-MAP kinase antibodies (Figure 7B).

Bacterial Adherence Is Sufficient for Induction of MAP Kinase

Cytochalasin D is a drug which disrupts actin filaments. It has also been shown to block *L. monocytogenes* uptake but not adherence (Gaillard *et al.*, 1987). MAP kinase tyrosine phosphorylation was still observed even when bacterial invasion was dramatically reduced by cytochalasin D (Figure 8A,B). Thus MAP kinase activation must occur before the actin polymerization required to

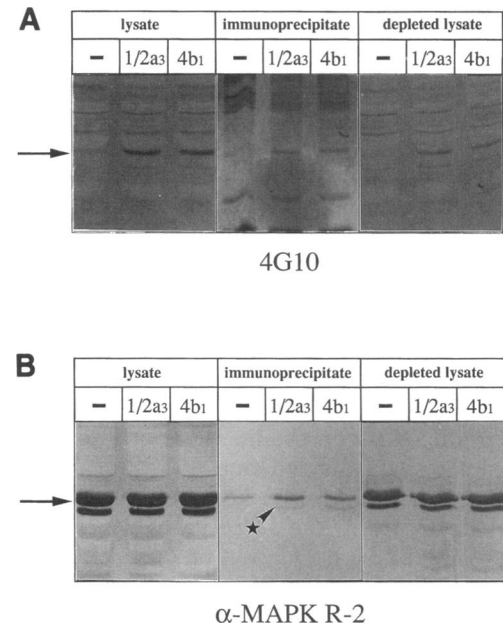


Figure 6. Immunoprecipitation of the 44 kDa protein. Lysates from uninfected HeLa cells (-), and HeLa cells infected with *L. monocytogenes* (1/2a₃ and 4b₁) were immunoprecipitated with anti-phosphotyrosine antibodies (4G10). (A) Immunoblot with 4G10. The arrow points to the position of the 44 kDa tyrosine phosphorylated protein seen in the lysate of infected cells. After 4G10 immunoprecipitation, it is detectable both in the immunoprecipitate and the depleted lysate of infected cells. (B) Immunoblot with anti-MAP kinase antibodies (α-MAPK R-2). The levels of p44 MAP kinase and p42 MAP kinase are equal in the lysates of infected and uninfected HeLa. However, the increased amount of p44 MAP kinase in the 4G10 immunoprecipitate of infected cell lysates suggests that p44 MAP kinase is tyrosine phosphorylated upon *L. monocytogenes* infection. The arrow shows the location of the p44 MAP kinase. The star (*) shows the position of p42 MAP kinase, which is also tyrosine phosphorylated in the infected samples.

uptake the bacteria. Also, using a noninvasive mutant, M44, MAP kinase was again activated in the absence of invasion (Figure 8A,C). These results clearly show that MAP kinase is being activated at the adherence stage of invasion prior to the actual internalization of the bacteria. This suggests that *L. monocytogenes* is activating a host cell surface receptor which directs the phosphorylation of MAP kinase. The activation of MAP kinase may be one factor required to initiate the uptake process. Other components must also be involved because the noninvasive M44 mutant is capable of activating MAP kinase but is not internalized.

As internalin has been shown to be required for *L. monocytogenes* adherence and invasion into host cells, we investigated its role in activating MAP kinase during invasion. The *inlA* mutant, BUG11, which is deficient in adherence and invasion does not trigger MAP kinase tyrosine phosphorylation, while its parent, EGD SmR (bof297) does (Figure 8A,D). The noninvasive *L. innocua* is also unable to induce phosphorylation of MAP kinase

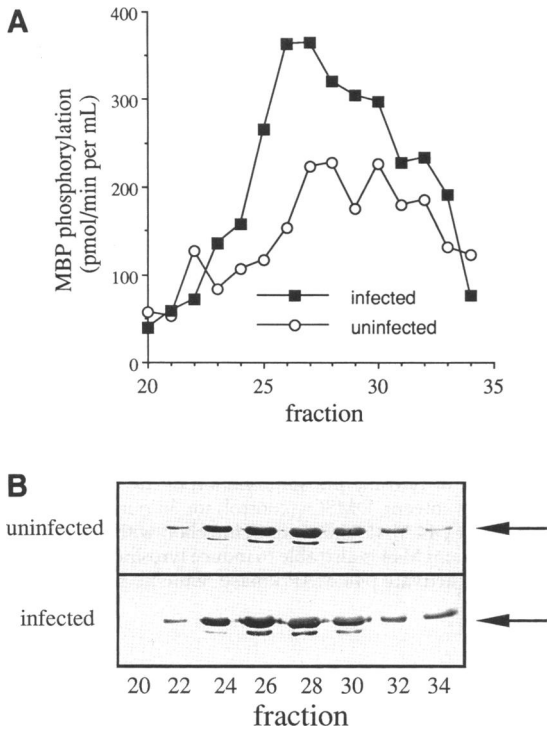


Figure 7. *L. monocytogenes* infection induces MBP kinase activity. (A) Lysates from uninfected HeLa cells and HeLa cells infected with *L. monocytogenes* (1/2a₃) were fractionated by anion exchange chromatography as described in MATERIALS AND METHODS. Fractions containing p42 and p44 MAP kinase were assayed for their ability to phosphorylate MBP. The samples from infected cells showed an increase in MBP phosphorylation over uninfected cells. (B) Immunoblots with anti-MAP kinase antibodies showing column fractions that contain p42 and p44 MAP kinase. The arrows show the location of p44 MAP kinase.

(Tang, Rosenshine, and Finlay, unpublished observations). As the *inlA* mutants and *L. innocua* do not adhere as efficiently as wild-type *L. monocytogenes*, it seems that efficient adherence is important for triggering MAP kinase phosphorylation. Whether internalin is the bacterial factor responsible for activating MAP kinase in the host cell remains to be determined.

DISCUSSION

Tyrosine phosphorylation of the p42 and p44 isoforms of MAP kinase was observed during invasion of *L. monocytogenes* into HeLa, Henle-407, and CaCo-2 epithelial cells. Immunoreactivity with anti-phosphotyrosine and anti-MAP kinase antibodies showed that the level of tyrosine phosphorylated MAP kinase in *L. monocytogenes* infected cells was elevated. The tyrosine phosphorylated MAP kinase also co-eluted with MBP phosphorylation activity following anion exchange chromatography indicating increased MAP kinase activity in infected cells. These results clearly show the

activation of MAP kinase by *L. monocytogenes*. This induced activation was also transient: the removal of invading bacteria led to the tyrosine dephosphorylation of MAP kinase.

MAP kinases play an essential role in intracellular signaling in eucaryotic cells (Pelech and Sanghera, 1992a,b; Davis, 1993; Nishida and Gotoh, 1993). MAP kinases are a group of serine/threonine kinases with isoforms ranging from 40 to 62 kDa. Many growth factors and differentiation factors use the MAP kinase signaling cascade to effect their actions: EGF, PDGF, NGF, insulin, and lymphokines have all been demonstrated to stimulate MAP kinase activity (Nakamura *et al.*, 1983; Cooper and Hunter, 1985; Kohno, 1985; Boulton *et al.*, 1991; Campos-Gonzalez and Glenney, 1991). MAP kinase is regulated by phosphorylation on a threonine and a tyrosine residue at the T-E-Y site: both residues must be phosphorylated for maximal activity (Anderson *et al.*, 1990; Payne *et al.*, 1991). Activated MAP kinases have been shown to phosphorylate several different substrates. MAP kinase can stimulate mitogenesis by translocating into the nucleus and phosphorylating transcription factors including c-Myc, NF-IL6, p62^{TCF}/Elf-1, ATF-2, and c-Jun (Pulverer *et al.*, 1991; Abdel-Hafiz *et al.*, 1992; Chou *et al.*, 1992; Gille *et al.*, 1992; Gupta *et al.*, 1993; Nakajima *et al.*, 1993). Another important target of MAP kinase is cytoplasmic phospholipase A₂ (PLA₂) (Lin *et al.*, 1992). Activation of PLA₂ mediates the release of arachidonic acid for the synthesis of leukotrienes. MAP kinase also functions during mitosis to initiate the microtubule rearrangements required for metaphase (Gotoh *et al.*, 1991). There is evidence that MAP kinase phosphorylates the microtubule associated protein tau to effect changes in microtubule dynamics in the cell (Drechsel *et al.*, 1992).

These diverse roles of MAP kinase suggest possible functions it may play during the uptake of bacteria. Galan and colleagues (1992) have observed the tyrosine phosphorylation of EGFR and MAP kinase during the invasion of *S. typhimurium* into Henle-407 cells (Pace *et al.*, 1993). The significance of this is unclear as tyrosine kinase inhibitors which block MAP kinase activation through the EGFR do not block *S. typhimurium* invasion (Rosenshine *et al.*, 1992b). Also, other invasive enteric bacteria including EPEC and *E. coli* expressing *inv* do not induce any observable tyrosine phosphorylation of MAP kinase in host epithelial cells. However, *E. coli* lipopolysaccharide (LPS) can activate MAP kinase in macrophages (Weinstein *et al.*, 1992). This activation involves the macrophage receptor CD14 and possibly mediates anti-bacterial responses in the macrophage. The *L. monocytogenes* induction of MAP kinase tyrosine phosphorylation is likely to be different from that reported for *S. typhimurium* as tyrosine kinase inhibitors do block *L. monocytogenes* invasion. In addition, *L. monocytogenes* are gram-positive and thus lack LPS, and the

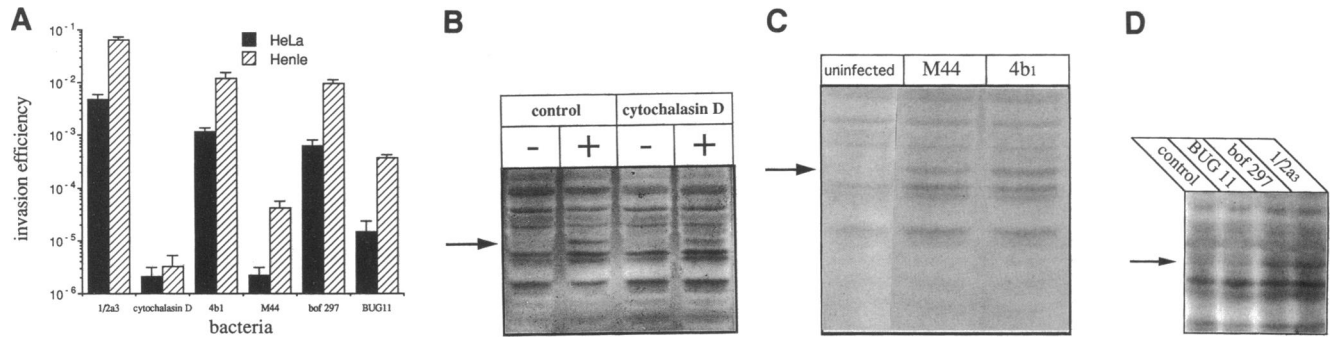


Figure 8. Bacterial internalization is not required for activation of p44 MAP kinase. (A) The invasion efficiencies of various strains of *L. monocytogenes* are expressed as the fraction of bacterial inoculum that was internalized by HeLa and Henle cells. Cytochalasin D (1 μ g/mL) pretreatment of cells for 30 min blocked the uptake of wild-type 1/2a₃. The noninvasive mutant M44 was significantly reduced in its ability to invade relative to its parent 4b₁. The *inlA* mutant, BUG11, was also reduced in its invasion efficiency relative to its wild-type parent EGD SmR (bof297). (b, c, and d) Anti-phosphotyrosine immunoblots of HeLa cell lysates. The arrows point to the location of tyrosine phosphorylated p44 MAP kinase. (B) Cytochalasin D inhibits bacterial internalization but not induction of tyrosine phosphorylation of the p44 MAP kinase. HeLa cells were pretreated with cytochalasin D (1 μ g/mL) or with an equal volume of its solvent, DMSO, (control) for 30 min. Cells were then infected with 1/2a₃ (+) or left uninfected (-). HeLa cells show tyrosine phosphorylation of p44 MAP kinase upon infection with *L. monocytogenes* even when cytochalasin D is used to block bacteria internalization. (C) The noninvasive mutant M44 is also able to induce tyrosine phosphorylation of p44 MAP kinase. (d) BUG11, which is deficient in adherence and invasion, does not activate p44 MAP kinase while its parent, EGD SmR (bof297) and the strain 1/2a₃ trigger p44 MAP kinase tyrosine phosphorylation.

HeLa, Henle and CaCo-2 epithelial cells do not possess the CD14 marker.

The ability of the tyrosine kinase inhibitor, genistein, to inhibit both *L. monocytogenes* induced tyrosine phosphorylation of MAP kinase and bacterial internalization suggests that protein tyrosine kinases play an important role in these two events. It is possible that the MAP kinase activation through tyrosine kinase activity is responsible for triggering the cytoskeletal changes necessary for uptake of *L. monocytogenes*. If MAP kinase is involved in directing *L. monocytogenes* internalization, the rapidity of invasion and MAP kinase tyrosine phosphorylation suggest that MAP kinase induced transcription and translation are too slow to direct the required cytoskeletal changes for bacterial uptake. It is possible that MAP kinase's other cellular functions such as microtubule organization and PLA₂ activation are important in phagocytosis of invasive bacteria (Galan *et al.*, 1992). When cytochalasin D was used to block bacteria internalization by disrupting host cell actin filaments, tyrosine phosphorylation of MAP kinase was still induced in infected cells. This shows that activation of MAP kinase occurs while the invading bacteria are still adhering extracellularly to the host cell. One possible scenario is the interaction of a bacterial ligand with a host cell surface receptor. The bound receptor then activates cytoplasmic components including MAP kinase which mediate the cytoskeletal changes required for bacterial uptake. One candidate for a bacterial ligand is the internalin surface protein which is involved in the attachment and invasion of *L. monocytogenes* into host cells: *inlA* mutants do not trigger MAP kinase tyrosine phosphorylation. Possible host receptors include the growth factor receptors and serpentine receptors

linked to trimeric G-proteins, both of which have been demonstrated to activate MAP kinase (Nishida and Gotoh, 1993; Ruderman, 1993).

However, the ability of a noninvasive mutant, M44, to induce tyrosine phosphorylation of MAP kinase suggests that this sequence of events may be more complicated. MAP kinase may be only one of several factors which must be activated for the invasion event, and M44 may be unable to stimulate one of these other host factors. The internalization process is possibly complex and involves more than one signal transduction pathway. While these hypotheses are consistent with the results of this study, the possibility that MAP kinase activation is independent of invasion cannot be ignored. MAP kinase activation may be triggered secondarily by the invasion signaling pathway or MAP kinase may be involved in directing other cellular responses to the invading bacteria.

Clearly, the participation of protein tyrosine kinases in *L. monocytogenes* invasion and MAP kinase activation is crucial. In similar studies with kinase inhibitors, EPEC and *E. coli* expressing *inv* were shown to require tyrosine kinase activity for invasion (Rosenshine *et al.*, 1992a,b). The involvement of host kinases in these bacterial invasion systems indicate that invasion is a cooperative event that requires communication between the bacteria and the host cell. It is possible that these different invading bacteria enlist certain common host components to enable their uptake into the host cell. On the premise that MAP kinase is part of the signal transduction pathway for *L. monocytogenes* invasion, we are currently searching for other components, upstream and downstream of MAP kinase activation that may function in bacterial uptake. This will hopefully give us a better

understanding of the host-parasite interactions required for the internalization of *L. monocytogenes* and other invasive bacteria.

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