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Listeriolysin O Activates Mitogen-Activated Protein Kinase in Eucaryotic Cells

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Infection with *Listeria monocytogenes* induces the activation of mitogen-activated protein (MAP) kinase in several tissue culture cell lines (P. Tang, I. Rosenshine, and B. B. Finlay, Mol. Biol. Cell 5:455–464, 1994). After various mutants were examined, the bacterial factor responsible for MAP kinase activation was identified as listeriolysin O (LLO). Growth supernatant containing LLO or purified LLO alone can induce MAP kinase tyrosine phosphorylation in HeLa cells. Single-amino-acid mutations in LLO that do not affect its membrane binding capacity but reduce its cytolytic activity also reduced its ability to induce MAP kinase activity in HeLa cells. Streptolysin O, another sulfhydryl-activated hemolysin, and the detergent saponin are also able to activate MAP kinase in target cells. Thus, the increased MAP kinase activity observed in *L. monocytogenes*-infected cells is most likely a result of the permeabilization of the host cell membrane by LLO and may not be linked with invasion.

Listeria monocytogenes is a gram-positive, facultative intracellular bacterium capable of causing severe infections in immunocompromised humans and animals (7, 17). Its pathogenicity is based on its ability to survive and replicate in the phagocytic cells (macrophages and monocytes) of its host (10). Virulent strains of *L. monocytogenes* are also capable of invading nonphagocytic cells, and these cells are thought to be the initial targets during the infection process (16). Recently, we reported tyrosine phosphorylation of mitogen-activated protein (MAP) kinase in *L. monocytogenes*-infected epithelial cells (18). MAP kinase is central in many host responses, including the mitogenic response after exposure to growth factors (13–15). MAP kinase has also been implicated in the regulation of cytokine responses and cytoskeletal organization (3, 5, 6, 12). With so many diverse roles for MAP kinase in the eucaryotic cell, the significance of its activation in *L. monocytogenes*-infected cells is unclear. In our previous paper, we speculated that MAP kinase played a role in triggering the host cell uptake of *L. monocytogenes* (18). In this report, we present evidence that the *L. monocytogenes* hemolysin, listeriolysin O (LLO), is responsible for inducing MAP kinase tyrosine phosphorylation in eucaryotic cells. Since LLO is not required for invasion (9), these results suggest that the significance of MAP kinase activation may be different from the significance we initially proposed.

Many virulence factors of pathogenic bacteria are secreted into the growth supernatant. To characterize the bacterial factor responsible for activating MAP kinase, we tested for its presence in the growth supernatant of logarithmic-phase *L. monocytogenes*. The epithelial cell line HeLa was cultured at 37°C with 5% CO_2 in Eagle's minimum essential medium (MEM) (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal calf serum (GIBCO) and penicillin (100 U/ml) and streptomycin (50 μg/ml) (GIBCO). *L. monocytogenes* $1/2a_3$, a streptomycin-resistant derivative of SLCC 5764, was grown on Trypticase-soy agar and brain heart infusion (BHI) broth (Difco). After bacterium-free growth supernatant from *L. monocytogenes* $1/2a_3$ grown to logarithmic phase in BHI broth at 37°C with aeration was added to the HeLa cell tissue culture medium, tyrosine phosphorylation of MAP kinase was observed in the HeLa cells (Fig. 1). Cell lysates and phosphotyrosine Western immunoblots were prepared as described previously (18). The tyrosine phosphorylation was a quick response occurring within 10 min of the addition of the growth supernatant. The same host cell response was elicited after the HeLa cells were infected for 30 min with live bacteria washed in phosphate-buffered saline (PBS) and resuspended in MEM without supplements (Fig. 1). This result suggests that the *L. monocytogenes* factor responsible for activating host cell MAP kinase is both cell associated and secreted into the growth medium.

All of the known virulence factors of *L. monocytogenes* are regulated by PrfA at the transcription level (1, 2, 4). If the MAP-kinase-activating factor is involved in invasion, it is likely to be controlled by PrfA. Indeed, after the *prfA* mutant (SLCC 53) was added to HeLa cells, it was unable to activate MAP kinase, while its parent NCTC 7973 was able to activate MAP kinase (Fig. 2A). This mutant is deficient in many virulence events, including adherence and invasion. In addition, the growth supernatant of parental strain NCTC 7973 was able to activate MAP kinase, while the growth supernatant of SLCC 53 was missing the activating factor (Fig. 2B). LLO is one of the virulence factors regulated by PrfA. M3 is a nonhemolytic Tn*916* mutant derived from strain $1/2a_3$ (8). After M3 bacteria were added to HeLa cells, MAP kinase was not tyrosine phosphorylated after a 30-min infection (Fig. 2C). Parental strain $1/2a_3$ activated MAP kinase as observed before. The growth supernatant of M3, which does not contain LLO, also cannot activate MAP kinase in HeLa cells (Fig. 2D). From these results, it is clear that LLO is essential for MAP kinase activation, but it may be acting alone or in concert with other factors to bring about this signalling event.

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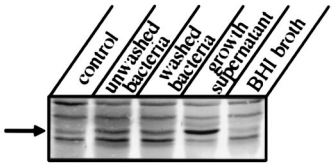

FIG. 1. Western immunoblot showing tyrosine-phosphorylated proteins from HeLa cells. *L. monocytogenes* $1/2a_3$ was grown to logarithmic phase in BHI broth and added directly to the HeLa cell monolayer or washed twice in PBS and resuspended in MEM prior to infection. HeLa cells were lysed at 30 min postinfection. Cell lysates were subjected to Western immunoblotting with anti-phosphotyrosine antibodies (4G10). Cells were also incubated in the logarithmic-phase growth supernatant or fresh BHI broth for 10 min. The control sample represents untreated cells. The arrow points to the position of tyrosine-phosphorylated MAP kinase.

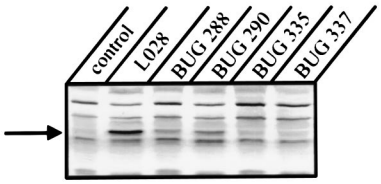

FIG. 3. Western immunoblot showing tyrosine-phosphorylated proteins from HeLa cells. Cells were treated with growth supernatants from *L. monocytogenes* L028 or from single-amino-acid mutants with mutations in *hly* (BUG 288, BUG 290, BUG 335, and BUG 337) for 10 min. The arrow indicates activated MAP kinase.

Next, we tried to determine the mechanism by which LLO activates MAP kinase in the host cells. Using the overnight stationary-phase growth supernatants from single-amino-acid mutants with mutations in *hly*, we found a correlation between cytolytic activity and the ability to activate MAP kinase (Fig. 3). BUG 288 (Cys-484 to Ser in the *hly* gene) and BUG 290 (Cys-484 to Ala) have 80 and 25% reductions in cytolytic activity, respectively, but the growth supernatants from both were still able to stimulate MAP kinase tyrosine phosphorylation in host cells. As was expected, the activation induced by the former was weaker. The growth supernatants of two other mutants with more dramatic reductions in cytolytic activity, BUG 335 (Trp-491 to Ala) with a 95% reduction and BUG 337 (Trp-492 to Ala) with a 99.9% reduction, were unable to activate MAP kinase at the same concentrations. The levels of secreted LLO were measured by Western immunoblotting with anti-LLO polyclonal antibodies and found to be the same for L028 and the mutants (data not shown). The LLOs from these mutants still bind cholesterol in the target cell membrane and are only reduced in their ability to form holes in the membrane (11).

LLO was purified by passing ammonium sulfate-precipitated growth supernatant from *L. monocytogenes* L028 through a DE-52 anion exchange column (Whatman) and then by gel filtration chromatography (Superdex 100; Pharmacia, Uppsala, Sweden). The fractions with the highest hemolytic activity were collected at each step (Fig. 4A). Hemolytic activity was measured as described previously (11). HeLa cells incubated with as little as 2 hemolytic units (HU) of LLO per ml for 10 min showed MAP kinase activation (Fig. 4B). At higher concentrations of LLO (50 HU/ml), cell lysis occurred, resulting in a reduced recovery of proteins (data not shown). Purified LLO from BUG 337 did not activate MAP kinase in HeLa cells, even after it was added at a concentration equivalent to 50 HU of normal LLO per ml (Fig. 5A).

Other agents capable of cytolysis were also tested for the ability to activate MAP kinase. HeLa cells treated with 50 HU of streptolysin O (Sigma) per ml from *Streptococcus pyogenes* similarly showed MAP kinase tyrosine phosphorylation (Fig. 5A). When the detergent saponin (Sigma) was added to the tissue culture medium, there was again an induction of MAP kinase tyrosine phosphorylation in the HeLa cells (Fig. 5B). This result suggests that any agent capable of permeabilizing the target cell membrane is able to trigger this host response. At higher concentrations of saponin, cell lysis occurred, resulting in lower protein recovery from the cells (Fig. 5B).

Our results show that tyrosine phosphorylation of MAP kinase during *L. monocytogenes* infection of host cells is a result of LLO. This effect is independent of invasion, as LLO does not play a role in *Listeria* invasion into host cells. In fact, purified LLO is sufficient for inducing the MAP kinase re-

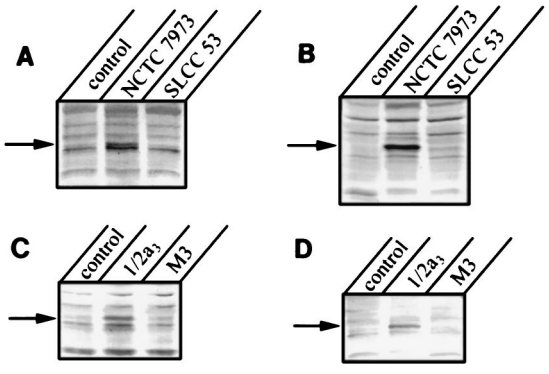

FIG. 2. Phosphotyrosine immunoblots of HeLa cell lysates. (A) Cells were infected with *L. monocytogenes* NCTC 7973 or its *prfA* mutant, SLCC 53, for 30 min. The control represents untreated cells. (B) Cells were treated with the logarithmic-phase growth supernatants from the two strains cited above or fresh BHI broth in MEM (control) for 10 min. (C) Cells were infected with wild-type *L. monocytogenes* $1/2a_3$ or its nonhemolytic mutant, M3, for 30 min. The control represents untreated cells. (D) Cells were treated with the logarithmic-phase growth supernatants from the two strains cited above or fresh BHI broth in MEM (control) for 10 min. The arrows point to the locations of tyrosine-phosphorylated MAP kinase.

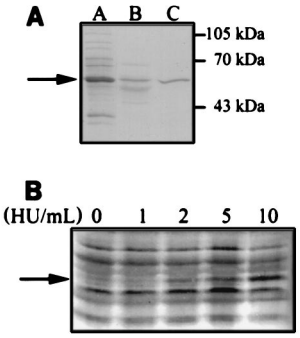

FIG. 4. Purified LLO from *L. monocytogenes* L028 and its effect on the HeLa cell tyrosine phosphorylation profile. (A) Coomassie brilliant blue-stained sodium dodecyl sulfate-polyacrylamide gel of samples at various stages of LLO purification. Lane A shows ammonium sulfate-precipitated growth supernatant. Lane B shows the sample after anion exchange chromatography. Lane C shows the LLO sample after gel filtration chromatography. The arrow points to the location of LLO. (B) Western immunoblot of tyrosine-phosphorylated proteins from HeLa cells. Cells were treated with different concentrations of purified LLO for 10 min. LLO was resuspended in 100 μl of PBS (pH 6.0) with 4 mM dithiothreitol before being added to the tissue culture medium. The control represents cells treated with only the buffer. The arrow points to the location of activated MAP kinase.

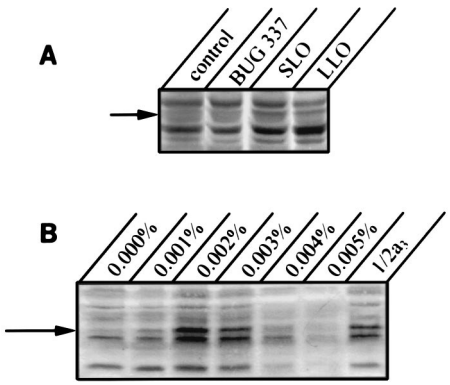

FIG. 5. Phosphotyrosine immunoblot of lysates from HeLa cells. (A) HeLa cells were treated for 10 min with wild-type LLO purified from L028, mutant LLO from BUG 337, and streptolysin O from *S. pyogenes*. Wild-type LLO was added to the HeLa cells at a final concentration of 10 HU/ml. The mutant LLO was added at a concentration equivalent to 50 HU of wild-type LLO per ml. Both were resuspended in 100 μl of PBS (pH 6.0) with 4 mM dithiothreitol before being added to the tissue culture medium. Streptolysin O was also added at a concentration of 50 HU/ml and resuspended in 100 μl of PBS (pH 7.4) with 4 mM dithiothreitol before being added to the tissue culture medium. The control represents cells treated with only the buffer. (B) HeLa cells were treated with 0 to 0.005% saponin in MEM for 10 min. The positive control represents cells treated with the logarithmic-phase growth supernatant from wild-type *L. monocytogenes* $1/2a_3$ for 10 min. The arrows point to the locations of activated MAP kinase.

sponse in target cells. The hemolytic activity of LLO is required for triggering this response, suggesting that host cell membrane damage by LLO leads to MAP kinase activation. Also, LLO is acting on the extracellular cytoplasmic membrane rather than the phagosomal membrane, as internalized bacteria do not activate MAP kinase: after the extracellular bacteria are removed, MAP kinase is dephosphorylated (18). Two other agents capable of cytolysis, the *S. pyogenes* cytolysin streptolysin O and the detergent saponin, were also able to activate MAP kinase. The implications of these results are that any membrane-damaging agent may activate MAP kinase. The significance of this signalling event is still unclear.

Previously, we showed that tyrosine kinase activity was required for *Listeria* invasion (18). The tyrosine kinase inhibitor genistein blocked both *Listeria* invasion and *Listeria*-induced MAP kinase tyrosine phosphorylation. Our present results show that these two effects are independent effects of genistein. While the tyrosine phosphorylation of MAP kinase may not be important for *Listeria* invasion, tyrosine phosphorylation of other yet unidentified host cell components is still crucial for the invasion process. Further studies examining the signals in pathogen-host cell interactions must be careful to address the possibility that there may be multiple signals with multiple responses in the host cell.

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REFERENCES

1. **Bohne, J., Z. Sokolovic, and W. Goebel.** 1994. Transcriptional regulation of *prfA* and PrfA-regulated virulence genes in *Listeria monocytogenes*. Mol. Microbiol. **11:**1141–1150.
2. **Chakraborty, T., M. Leimeister-Wächter, E. Domann, M. Hartl, W. Goebel, T. Nichterlein, and S. Notermans.** 1992. Coordinate regulation of virulence genes in *Listeria monocytogenes* requires the product of the *prfA* gene. J. Bacteriol. **174:**568–574.
3. **Davis, R. J.** 1993. The mitogen-activated protein kinase signal transduction pathway. J. Biol. Chem. **268:**14553–14556.
4. **Dramsi, S., C. Kocks, C. Forestier, and P. Cossart.** 1993. Internalin-mediated invasion of epithelial cells by *Listeria monocytogenes* is regulated by the bacterial growth state, temperature and the pleiotropic activator *prfA*. Mol. Microbiol. **9:**931–941.
5. **Drechsel, D. N., A. A. Hyman, M. H. Cobb, and M. W. Kirschner.** 1992. Modulation of the dynamic instability of tubulin assembly by the microtubule-associated protein tau. Mol. Biol. Cell **3:**1141–1154.
6. **Gotoh, Y., E. Nishida, S. Matsuda, N. Shiina, H. Kosako, K. Shiokawa, T. Akiyama, K. Ohta, and H. Sakai.** 1991. In vitro effects on microtubule dynamics of purified Xenopus M phase-activated MAP kinase. Nature (London) **349:**251–254.
7. **Gray, M. L., and A. H. Killinger.** 1966. *Listeria monocytogenes* and listeric infections. Bacteriol. Rev. **30:**309–382.
8. **Kathariou, S., P. Metz, H. Hof, and W. Goebel.** 1987. Tn*916*-induced mutations in hemolysin determinant affecting virulence of *Listeria monocytogenes*. J. Bacteriol. **169:**1291–1297.
9. **Kuhn, M., S. Kathariou, and W. Goebel.** 1988. Hemolysin supports survival but not entry of the intracellular bacterium *Listeria monocytogenes*. Infect. Immun. **56:**79–82.
10. **Mackaness, G. B.** 1962. Cellular resistance to infection. J. Exp. Med. **116:** 381–406.
11. **Michel, E., K. A. Reich, R. Favier, P. Berche, and P. Cossart.** 1990. Attenuated mutants of the intracellular bacterium *Listeria monocytogenes* obtained by single amino acid substitutions in listeriolysin O. Mol. Microbiol. **4:**2167–2178.
12. **Nakajima, T., S. Kinoshita, T. Sasagawa, K. Sasaki, M. Naruto, T. Kishimoto, and S. Akira.** 1993. Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6. Proc. Natl. Acad. Sci. USA **90:**2207–2211.
13. **Nishida, E., and Y. Gotoh.** 1993. The MAP kinase cascade is essential for diverse signal transduction pathways. Trends Biochem. Sci. **18:**128–131.
14. **Pelech, S. L., and J. S. Sanghera.** 1992. MAP kinases: charting the regulatory pathways. Science **257:**1355–1356.
15. **Pelech, S. L., and J. S. Sanghera.** 1992. Mitogen-activated protein kinases: versatile transducers for cell signaling. Trends Biochem. Sci. **17:**233–238.
16. **Rascz, P., K. Tenner, and E. Mero.** 1972. Experimental *Listeria* enteriditis: an electron microscopic study of the epithelial phase in experimental *Listeria* infections. Lab. Invest. **26:**694–700.
17. **Seeliger, H. P. R.** 1961. Listeriosis. Hafner Publishing Co., New York.
18. **Tang, P., I. Rosenshine, and B. B. Finlay.** 1994. *Listeria monocytogenes*, an invasive bacterium, stimulates MAP kinase upon attachment to epithelial cells. Mol. Biol. Cell **5:**455–464.