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9. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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A Role for Phosphoinositide 3-Kinase in Bacterial Invasion

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Listeria monocytogenes is a bacterial pathogen that invades cultured nonphagocytic cells. Inhibitors and a dominant negative mutation were used to demonstrate that efficient entry requires the phosphoinositide (PI) 3-kinase p85α-p110. Infection with *L. monocytogenes* caused rapid increases in cellular amounts of PI(3,4)P_2 and PI(3,4,5)P_3, indicating that invading bacteria stimulated PI 3-kinase activity. This stimulation required the bacterial protein InlB, host cell tyrosine phosphorylation, and association of p85α with one or more tyrosine-phosphorylated proteins. This role for PI 3-kinase in bacterial entry may have parallels in some endocytic events.

Listeria monocytogenes, a bacterium that causes severe food-borne infections in humans, penetrates or invades nonphagocytic cells in the intestine and other organs (*1*). Invasion into the intestinal epithelial cell line Caco-2 is mediated by interaction between a bacterial surface protein and a cellular receptor, the adhesion molecule E-cadherin (*2*). Little is known, however, about host proteins that affect the entry process after the initial interaction between the bacterium and its receptor. Inhibition of tyrosine phosphorylation (*3*) or actin polymerization (*1*) impairs invasion, but proteins controlling phosphorylation or the actin cytoskeleton during entry have not been identified.

One signaling protein implicated in actin polymerization and activated upon receptor stimulation and tyrosine phosphorylation is the phosphoinositide (PI) 3-kinase p85-p110 (*4*). This lipid kinase is composed of an 85-kD regulatory subunit (p85) and a 110-kD catalytic subunit (p110), each of which exists in at least two isoforms (α and β). The p110 protein phosphorylates the D-3 position of the inositol ring of PI, PI 4-phosphate, and PI 4,5-bisphosphate, which are found in membranes. Two of the resulting products, PI(3,4)P_2 and PI(3,4,5)P_3, may act as second messengers; these lipids are virtually absent in quiescent cells, rapidly increase in amount upon stimulation, and appear to regulate protein kinases or other targets (*4*). In addition to p85-p110, mammalian cells contain at least two other classes of PI 3-kinases (*5*). Recently, PI-3 kinase activity has been implicated in protein trafficking and some endocytic events (*4*, *5*), raising the possibility that a PI 3-kinase may participate in processes resembling endocytosis, such as the induced uptake of pathogens into nonphagocytic cells.

The structurally unrelated compounds wortmannin and LY294002 inhibit PI 3-kinase activity by different mechanisms (*6*, *7*). We examined these inhibitors for effects on entry of *L. monocytogenes* into several mammalian cell lines, including the human epithelial cell lines Caco-2 and HeLa, and the African green monkey kidney cell line Vero. Vero cells were the most sensitive, with half-maximal inhibition of entry occurring at concentrations close to the median inhibitory concentrations (IC_{50}) of these compounds in vitro—about 5 nM for wortmannin and 5 μM for LY294002 (Fig. 1, A and B). At 50 nM, wortmannin reduced entry to about 1 to 2% of that into control dimethyl sulfoxide (DMSO)–treated cells, whereas 50 μM LY294002 reduced entry to about 10 to 20% of the control value. The more efficient inhibition by wortmannin is consistent with the relative effects of these inhibitors on PI 3-kinase activity in other systems (*8*). Entry into Caco-2 (Fig. 1C) and HeLa cells (*9*) was also impaired by wortmannin and LY294002, although the IC_{50} values were higher and the magnitude of inhibition lower than that for Vero cells. Inhibition appeared relatively specific to entry of *L. monocytogenes*, because wortmannin (50 nM) and LY294002 (50 μM) mildly stimulated entry of the unrelated invasive bacterium *Salmonella typhimurium* (strain SL1344) into Vero cells (*9*).

Wortmannin inhibits all three known classes of mammalian PI-3 kinases (*5*, *6*). To examine specifically whether p85α-p110 is needed for invasion, we used a dominant negative form of p85α (Δp85α) that inhibits PI-3 kinase activation by insulin and other agonists (*10*). Entry of *L. monocytogenes* into Chinese hamster ovary (CHO) cells stably overexpressing Δp85α (*11*) (0.012 ± 0.005% invasion) was about

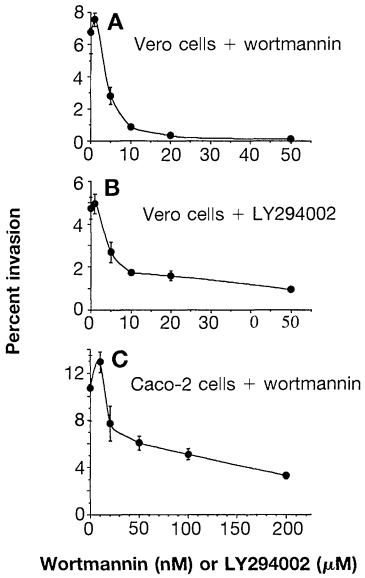

Fig. 1. Dose-dependent inhibition of entry of *L. monocytogenes* strain EGD by wortmannin and LY294002. Invasion tests into Vero or Caco-2 cells were done as described (*2*, *18*), and percent invasion values are the mean ± SD of triplicate (**A** and **B**) or duplicate (**C**) samples from a single experiment. Similar results were obtained in at least two other experiments.

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one-tenth of that into the parental CHO cell line (0.15 ± 0.08% invasion) (*12*). These results indicate that a PI-3 kinase, most likely the p85α-p110 heterodimer, is required for entry of *L. monocytogenes* into cultured cells.

Infection of cells with *L. monocytogenes* rapidly stimulated PI 3-kinase activity. In uninfected Vero cells, ^{32}P-labeled PI(3,4)P_2 and PI(3,4,5)P_3 were undetectable or barely detectable [less than 0.05 and 0.20% of the amount of labeled PI(4,5)P_2, respectively], and infection for 5 min caused approximately 10- and 20-fold increases in the amounts of these two products (Fig. 2, A and B). These increases were inhibited by wortmannin (50 nM) (Fig. 3A) and LY294002 (80 μM) (9) at concentrations that impaired entry (Fig. 1). In contrast to PI(3,4)P_2 and PI(3,4,5)P_3, the amount of labeled PI(4,5)P_2 was unaffected or only slightly increased by infection (Fig. 2C).

Treatment of Vero or other cells with cytochalasin D (5 μg/ml) or the tyrosine kinase inhibitor genistein (250 μM) inhibits entry but not adherence of *L. monocytogenes* (*1*, *3*, 9), indicating that reorganization of the actin cytoskeleton and tyrosine phosphorylation, respectively, are needed for invasion. However, treatment of Vero cells with cytochalasin D did not prevent bacterially induced increases in labeled PI(3,4,5)P_3 (Fig. 3A), demonstrating that rearrangement of the actin cytoskeleton is not needed for stimulation of PI-3 kinase. This result also suggests that PI-3 kinase may act before cytoskeletal changes during entry. In contrast, treatment of cells with genistein did inhibit increases in labeled PI(3,4,5)P_3 (Fig. 3A), indicating that tyrosine phosphorylation is required for stimulation of PI-3 kinase activity by *L. monocytogenes*.

Tyrosine phosphorylation is also needed for activation of p85α-p110 in several growth factor signaling pathways. Activation results from interaction between the SRC homology 2 (SH2) domains of p85α and tyrosine-phosphorylated residues of a key membrane-associated protein (*4*), and such interactions are often demonstrated by coimmunoprecipitation with antibodies to phosphotyrosine (*8*). Infection with *L. monocytogenes* also appeared to stimulate association of p85α with one or more tyrosine-phosphorylated proteins. In uninfected Vero cells, amounts of p85α were barely detectable after immunoprecipitation with antibodies to phosphotyrosine and greatly increased after bacterial infection (Fig. 4). We were unable to detect tyrosine phosphorylation of p85α (9), indicating that immunoprecipitation did not result from phosphorylation of p85α itself. The amount of p85α immunoprecipitated was reduced by treatment with genistein, but not with cytochalasin D (9) or wortmannin (Fig. 4).

At least two surface proteins of *L. monocytogenes* mediate entry into mammalian cells. InlA (internalin A) is needed for entry into Caco-2 cells (*13*, *14*), whereas InlB is required for entry into other cell lines including Vero, CHO, HeLa (9), and some cultured hepatocytes (*13*). InlB is also needed for efficient stimulation of PI-3 kinase activity in Vero cells. An *inlB* deletion mutant (*13*) invaded these cells at about 1% the efficiency of the isogenic wild-type strain and was defective in increasing labeled PI(3,4,5)P_3 (Fig. 3B) and in stimulating association of p85α with tyrosine-phosphorylated proteins (Fig. 4).

The role of PI-3 kinase in InlA-mediated invasion was also examined. Expression of InlA is sufficient to cause the normally noninvasive bacterium *Listeria innocua* to invade Caco-2 cells or other cell lines expressing the

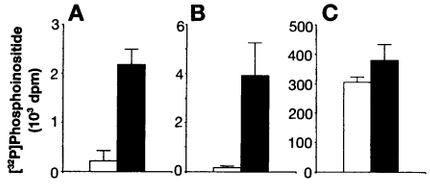

Fig. 2. Effects of infection with *L. monocytogenes* on amounts of cellular PI(3,4)P_2 (**A**), PI(3,4,5)P_3 (**B**), and PI(4,5)P_2 (**C**). Vero cells (0.6 × 10^6 to 1 × 10^6) were seeded in 75-cm^2 flasks, grown at 37°C in 5% CO_2 for 40 to 46 hours, and labeled with [^{32}P]*o*-phosphate (250 μCi/ml, Amersham) for 4.5 to 7 hours in DMEM without phosphate (ICN) supplemented with glutamine and 0.5% fetal calf serum. Cells were then washed twice with DMEM and either incubated in 10 ml of DMEM or infected for 5 min with 10 ml of a bacterial suspension of strain EGD in DMEM (1 × 10^8 bacteria per milliliter; corresponding to a MOI of 3 to 400:1) (*20*). After infection, cells were washed with phosphate-buffered saline (PBS), reactions were stopped by adding ice-cold HCl (2.4 N), and cells were recovered by scraping. Lipids were then extracted (*16*), separated by thin-layer chromatography, and radiolabeled PI(3,4)P_2 + PI(4,5)P_2 and PI(3,4,5)P_3 were recovered by scraping the appropriate bands. Separation and analysis of deacylated products by high-performance liquid chromatography were as described (*16*). The data (from three independent experiments) are average disintegrations per minute per flask ± SEM of the particular ^{32}P-labeled phosphoinositide in uninfected control cells (□) or cells infected with EGD (■).

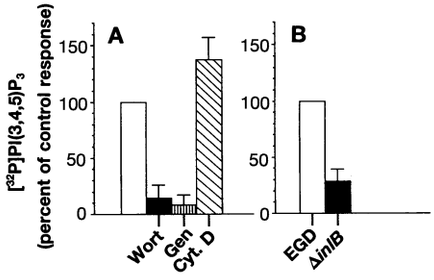

Fig. 3. Host and bacterial factors affecting PI 3-kinase activity. (**A**) Effects of inhibitors of PI 3-kinase (wortmannin), tyrosine kinases (genistein), and actin polymerization (cytochalasin D). Vero cells were grown and labeled with [^{32}P]*o*-phosphate as described (Fig. 2), treated with wortmannin (Wort, 50 nM) (□), genistein (Gen, 250 μM) (▥), cytochalasin D (Cyt. D, 5 μg/ml) (▨), or with the solvent DMSO (0.1%) (■) for 20 min and infected for 5 min with strain EGD. Inhibitors or DMSO were also included during the 5-min infection. (**B**) Requirement of the bacterial surface protein InlB. [^{32}P]*o*-phosphate–labeled Vero cells were infected either with the wild-type EGD (■) or with the isogenic Δ*inlB* mutant (□) for 5 min. Lipids were extracted and analyzed as described (Fig. 2). Data are expressed as percent of the response in infected DMSO-treated cells, where the 100% response is defined as the number of disintegrations per minute of ^{32}P-labeled PI(3,4,5)P_3 in the flask infected with EGD minus the number of disintegrations per minute of ^{32}P-labeled PI(3,4,5)P_3 in the control (uninfected) flask. All values are averages ± SEM from three experiments, except for those with genistein, which are from two experiments.

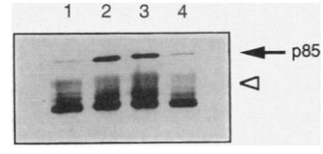

Fig. 4. Invasion stimulates association of p85α with tyrosine-phosphorylated proteins. Lane 1, uninfected cells; lane 2, cells infected with strain EGD; lane 3, cells treated with wortmannin and infected with EGD; and lane 4, cells infected with the Δ*inlB* mutant. Growth and infection of Vero cells was as described (Fig. 2), except that cells were not radiolabeled. Cells were treated with 0.1% DMSO (lanes 1, 2, and 4) or 50 nM wortmannin (lane 3) 20 min before infection and during the infection period (10 min). After infection, cells were washed with PBS, incubated in lysis buffer [50 mM NaCl, 50 mM tris-HCl (pH 8), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM EGTA, 3 mM sodium vanadate, and 5 μg/ml each of leupeptin and aprotinin], recovered by scraping, and centrifuged (10 min, 11,000*g*) to remove nuclei and cell debris. Immunoprecipitations with monoclonal antibody 4G10 (UBI) to phosphotyrosine and protein A–Sepharose CL-4B beads (Pharmacia) were as described (*16*). Samples were subjected to electrophoresis (7.5% SDS–polyacrylamide gel electrophoresis), transferred to nitrocellulose, probed with a polyclonal antibody to p85α (UBI), and detected with horseradish peroxidase–conjugated secondary antibodies to rabbit immunoglobulin G and the ECL system (Amersham). Shown is a protein immunoblot from one experiment, representative of three. The arrow indicates p85α and the open arrowhead shows signals from the immunoglobulin heavy chain.

receptor for InlA, E-cadherin (*2*, *13*, *14*). Entry of *L. innocua* expressing *inlA* into Caco-2 cells was inhibited by nanomolar or micromolar concentrations of wortmannin or LY294002, respectively (*9*), indicating that PI-3 kinase activity is needed for efficient InlA-mediated entry. However, in Caco-2 cells we failed to detect an increase in ^{32}P-labeled PI(3,4)P_2 or PI(3,4,5)P_3 upon infection with *L. monocytogenes* (*9*), suggesting that InlA does not stimulate PI-3 kinase activity. Interestingly, uninfected Caco-2 cells contained PI(3,4)P_2 and PI(3,4,5)P_3 in amounts [about 1 and 4% the amount of PI(4,5)P_2, respectively] that were considerably greater than those in uninfected Vero cells. These results suggest that Caco-2 cells have higher basal PI-3 kinase activity than Vero cells and raise the possibility that InlA–E-cadherin–mediated entry requires that host PI 3-kinase be constitutively activated.

It is not known how activation of p85α-p110 affects bacterial invasion, but one possibility is through reorganization of the actin cytoskeleton. PI(3,4)P_2 and PI(3,4,5)P_3 (and D-4–phosphorylated phosphoinositides) uncap barbed actin filaments in permeabilized platelets (*15*), suggesting that bacterial stimulation of PI 3-kinase might drive local changes in the cytoskeleton by directly affecting actin polymerization. In addition, p85α-p110 can interact with proteins that regulate the actin cytoskeleton, such as pp125^{FAK} (*16*) or rho guanosine triphosphatases (GTPases) (*4*), and such interactions could participate in invasion. PI 3-kinase activity also appears to be involved in phagocytosis and some endocytic processes (*4*, *5*), and p85α can interact with the GTPase dynamin (*17*), an essential component of clathrin-dependent endocytosis. It is possible that dynamin or other components of endocytosis are also used by invasive pathogens to enter host cells. Understanding the function of PI 3-kinase in bacterial uptake might therefore help elucidate the roles of this lipid kinase in certain endocytic processes.

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12. Percent invasion values are the means ± SD of four experiments. Construction of CHO-Δp85α cells will be described elsewhere (*11*). CHO and CHO-Δp85α cells were grown as described (*10*) and used between passages 4 to 8 and 5 to 8, respectively. Hygromycin (150 ng/ml) was included in the medium for routine growth of CHO-Δp85α cells, except when seeded for invasion tests. Invasion assays were performed on 2-day-old monolayers in 24-well tissue culture plates (about 4 × 10^4 cells per well). Expression of Δp85α did not affect bacterial adhesion, or cell viability, as determined by Trypan blue staining.
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18. Vero cells were grown in Dulbecco's modified Eagle's medium (DMEM) with fetal calf serum (10%) (Gibco) and Non-Essential Amino Acids (Gibco). Caco-2 cells were grown as described (*13*) and used between passages 78 and 86. Subconfluent monolayers of cells for invasion tests were obtained by seeding 2 × 10^4 Vero or 4 × 10^4 Caco-2 cells per well in 24-well tissue culture plates and incubating at 37°C in 10% CO_2 for 2 days before the test. Strain EGD (*13*) was prepared for invasion tests as described (*2*), and a multiplicity of infection (MOI) of about 100 bacteria per cell was used. Vero or Caco-2 cells were treated with wortmannin (Sigma or Biomol) or LY294002 (Biomol) 20 min before infection, as well as during the 60-min infection. Inhibitors were added from 1000× solutions in DMSO, and therefore DMSO was added at a final concentration of 0.1% in the control (0 concentration). At the concentrations used, the inhibitors had no effect on cell or bacterial viability as assayed by the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT)] test (*19*) or titering of viable bacteria, respectively. The inhibitors also did not affect bacterial adhesion to Vero or Caco-2 cells.
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An Essential Role for NF-κB in Preventing TNF-α–Induced Cell Death

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Studies on mice deficient in nuclear factor kappa B (NF-κB) subunits have shown that this transcription factor is important for lymphocyte responses to antigens and cytokine-inducible gene expression. In particular, the RelA (p65) subunit is required for induction of tumor necrosis factor–α (TNF-α)–dependent genes. Treatment of RelA-deficient (RelA$^{-/-}$) mouse fibroblasts and macrophages with TNF-α resulted in a significant reduction in viability, whereas RelA$^{+/+}$ cells were unaffected. Cytotoxicity to both cell types was mediated by TNF receptor 1. Reintroduction of RelA into RelA$^{-/-}$ fibroblasts resulted in enhanced survival, demonstrating that the presence of RelA is required for protection from TNF-α. These results have implications for the treatment of inflammatory and proliferative diseases.

The transcription factor NF-κB is a critical regulator of cytokine-inducible gene expression (*1*). Pro-inflammatory cytokines, such as TNF-α and interleukin-1 (IL-1), allow rapid nuclear translocation of NF-κB through degradation of IκB inhibitory cytoplasmic retention proteins (*1*, *2*). Genes regulated by nuclear NF-κB include those involved in inflammatory responses such as hematopoietic growth factors, chemokines, and leukocyte adhesion molecules (*1*). Thus, NF-κB is a key mediator of TNF-α responses and an attractive target for therapeutic intervention against inflammation and inflammatory diseases such as rheumatoid arthritis.

The most common form of NF-κB is a heterodimer of a 50-kD protein (p50) and a 65-kD protein (RelA, also termed p65) (*3*–*6*). As expected, embryonic fibroblasts (EFs) derived from RelA$^{-/-}$ embryos are unable to transcriptionally activate certain TNF-α–inducible genes with NF-κB binding sites (*7*), although p50$^{-/-}$ EFs show normal activation (*8*, *9*). TNF-α is not only involved in inflammatory responses but also possesses considerable cytotoxic activity, especially against tumor cells (*10*–*12*). The inability of RelA$^{-/-}$ EFs to activate target genes prompted us to investigate whether long-term TNF-α treatment leads to alterations in cellular function. An 8-hour treatment of RelA$^{-/-}$ EFs with mouse TNF-α (mTNF-α) resulted in a dramatic decrease in viability (Fig. 1A) (*13*). Longer treat-

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