The Gram-positive pathogen *Listeria monocytogenes* induces its own internalization into some non-phagocytic mammalian cells by stimulating host tyrosine phosphorylation, phosphoinositide (PI) 3-kinase activity, and rearrangements in the actin cytoskeleton. Entry into many cultured cell lines is mediated by the bacterial protein InlB. Here we investigate the role of InlB in regulating mammalian signal transduction and cytoskeletal structure. Treatment of Vero cells with purified InlB caused rapid and transient increases in the lipid products of the PI 3-kinase p85-p110, tyrosine phosphorylation of the mammalian adaptor proteins Gab1, Cbl, and Shc, and association of these proteins with p85. InlB also stimulated large scale changes in the actin cytoskeleton (membrane ruffling), which were PI 3-kinase-dependent. These results identify InlB as the first reported non-mammalian agonist of PI 3-kinase and demonstrate similarities in the signal transduction events elicited by this bacterial protein and known agonists such as epidermal growth factor.

Infection of viral, bacterial, or protozoan intracellular pathogens often involves subversion of mammalian signaling pathways. The first step in the life cycle of an intracellular pathogen is internalization by the host cell. While some microbial pathogens are taken up only by professional phagocytes (macrophages or neutrophils), others induce their own uptake (“enter”) into cells that are not normally phagocytic, such as epithelial cells underlying mucosal surfaces or endothelial cells inside blood vessels (reviewed in Ref. 1). Entry into non-phagocytic cells can permit traversal of tissue-specific barriers and promote survival of the pathogen by providing access to a nutrient-rich environment that is protected from host antibody or complement.

*Listeria monocytogenes* is a food-borne bacterial pathogen that causes severe illnesses leading to meningitis or abortions in immunocompromised individuals or pregnant women (2).

This facultative intracellular pathogen enters into cells that are non-phagocytic, including epithelial cells and hepatocytes. Entry into such cells is likely to play an important role in traversing the intestinal, blood-brain, and placental barriers, and in colonization of the liver (reviewed in Ref. 3).

Entry of *L. monocytogenes* into non-phagocytic cells involves specific bacterial surface proteins that exhibit cell tropism. The bacterial protein InlA (internalin) promotes entry into the intestinal epithelial cell line Caco-2, whereas the protein InlB mediates internalization into several other cultured cell lines, including Vero, HEp-2, HeLa, and some hepatocytes (4–6). InlB has a role in virulence in the mouse model, as a bacterial mutant (ΔinlB) deleted for the inlB gene is defective in colonization of the liver (4, 5, 7). InlA promotes bacterial entry by interacting with its mammalian receptor, the cell-cell adhesion molecule E-cadherin (8). The mammalian receptor for InlB is not known.

Entry of *L. monocytogenes* requires tyrosine phosphorylation and other signal transduction events that are likely to occur downstream of engagement of E-cadherin or the InlB receptor (3, 9). One of the ultimate effects of such signaling is to promote rearrangements in the actin cytoskeleton that drive the entry process. One of the mammalian signaling proteins that controls bacterial entry is the phosphoinositide (PI) 3-kinase p85-p110 (6). Infection with *L. monocytogenes* induces accumulation of PtdIns(3,4)P2 and PtdIns(3,4,5)P3 in Vero cells, indicating that this bacterium activates PI 3-kinase. Activation of p85-p110 is required for bacterial uptake, since entry is blocked by genetic or pharmacological inhibition of p85-p110. Bacterial stimulation of PI 3-kinase is accompanied by interaction of p85 with one or more tyrosine phosphorylated host proteins, indicating that tyrosine phosphorylation is involved in signaling mediated by p85-p110. The specific phosphoproteins that associate with p85 have not been identified.

p85-p110 is a heterodimeric lipid kinase, composed of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit, which is activated in response to engagement of cell surface receptors by some growth factors or cytokines. Activation of p85-p110 is often promoted by recruitment to the plasma membrane through interaction of SH2 domains in p85 with tyrosine-phosphorylated proteins (10). Activation of p85-p110 and some other PI 3-kinases in *vivo* is characterized by rapid and often transient increases in levels of PtdIns(3,4)P2 and PtdIns(3,4,5)P3, phosphoinositides that appear to act as membrane-bound second messengers to regulate the cellular local-

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**The Listeria monocytogenes Protein InlB Is an Agonist of Mammalian Phosphoinositide 3-Kinase**

(Received for publication, February 19, 1999, and in revised form, March 26, 1999)

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This paper is available on line at http://www.jbc.org
A Bacterial Activator of PI 3-Kinase

ization and/or activity of various signaling proteins (11). p85-p110 controls several critical processes in mammalian cells, including cell growth, vesicular trafficking, apoptosis, and organization of cytoskeletal actin. Although the function of p85-p110 in entry of *L. monocytogenes* is not understood, an attractive possibility is that this kinase controls cytoskeletal changes needed for bacterial uptake.

In this report, we identify one of the bacterial factors that activates PI 3-kinase in mammalian cells. A recombinant InlB protein stimulated accumulation of the lipid products of p85-p110, tyrosine phosphorylation of three mammalian adaptor proteins, and the association of these adaptor proteins with p85. InlB also provoked PI 3-kinase-dependent membrane ruffling. These findings establish InlB as the first reported non-mammalian agonist of p85-p110 and suggest similarities between signal transduction promoted by this bacterial protein and some mammalian growth factors.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Media**—The African green monkey kidney cell line Vero was grown at 37 °C in 5 or 10% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco-BRL) or 10% fetal calf serum (FCS), 2 mM glutamine, and non-essential amino acids. All experiments involving bacterial infections or treatments of cells with InlB or EGF were performed at 37 °C in 5 or 10% CO2.

**Antibodies, Inhibitors, and Other Materials**—Polyclonal antiserum against rat p85 (60-195), polyclonal antibodies against a peptide in human p85 (60-579), and monoclonal antibodies against phosphorylated (Tyr(P)) (clone 4G10; 16-101) were used for immunoprecipitation or treatment of cells with InlB or EGF were performed at 37 °C in 5 or 10% CO2. Antibodies, inhibitors, and other materials were purchased from Upstate Biotechnology. Affinity-purified polyclonal antibodies were raised against peptides in human Cbl (sc-170) and human epidermal growth factor receptor (EGF-R) (sc-03) were from Santa Cruz Biotechnology. Rabbit preimmune IgG was from Sigma. Monoclonal anti-Tyr(P) antibodies (clone RC20) coupled to peroxidase (E120H), the epidermal growth factor receptor (EGF-R) (sc-03) were from Santa Cruz Biotechnology. Affinity-purified polyclonal anti-IgG antibodies (115-0650) were from Transduction Laboratories.

Western blotting indicated that the anti-IgG antibodies recognized the three known Cbl isoforms (p46, p52, and p66) in Vero cells. Protein A-Sepharose CL-4B beads were from Amersham Pharmacia Biotech. The BCA kit used to determine protein concentrations of InlB preparations and solubilized mammalian lysates was from Pierce. Recombinant human epidermal growth factor was from Calbiochem or Upstate Biotechnology. Wortmannin was purchased from Biomol and Sigma, and the tyrosine kinase inhibitor AG1478 was from Calbiochem.

At the concentrations used, these inhibitors had no effect on the viability of Vero cells, as determined by staining with trypan blue.

**Purification of InlB**—We constructed an expression plasmid by polymerase chain reaction amplification of DNA containing the inlB coding region described above and cloned it into the expression vector pET28 (+) (Novagen), to yield plasmid pK122. pK122, verified by DNA sequencing, allows expression of a recombinant form of InlB that contains a 6His tag at the N-terminal of the protein. Cleavage of the recombinant protein with thrombin is predicted to yield a protein that is identical to full-length mature InlB (i.e., lacking the signal peptide) with the amino acids GSHMAS added to the NH2 terminus.

Immunoprecipitation and Western Blotting—Approximately 8 × 10^5 Vero cells were seeded in 75-cm^2 tissue culture flasks or 10-cm plates and grown for 40 h. Cells were then starved for 5 h in serum-free DMEM and treated with InlB or EGF or infected with *L. monocytogenes* strain EGD (BUG600). Cell lysates or supernatants were analyzed by immunoprecipitation or immunofluorescence using an antibody against a peptide in the EGF-R (sc-03) or a monoclonal antibody against the EGF-R (clone RC20).

InlB retains some activity after several rounds of freeze-thawing. Measurements of in vivo levels of PtdIns(3,4)P2 and PtdIns(3,4,5)P3—Approximately 8 × 10^5 Vero cells were seeded in 10-cm tissue culture dishes (78.5 cm^2 in surface area) and grown in DMEM with 10% fetal calf serum for approximately 30 h. Cells were then transfected with high molecular weight plasmid DNA containing DMECM with 0.2% fetal calf serum and labeled for 5 h by incubation in serum-free DMECM without phosphate (Sigma) containing 200 μM DlP42. Cells were left untreated or stimulated by addition of InlB or EGF to the indicated concentrations and incubation for the indicated times. When appropriate, wortmannin or the solvent Me2SO was added 20 min before treatment with InlB. After stimulation, cells were washed once with cold phosphate-buffered saline, reactions were stopped by addition of cold 2.4 M HCl, and cells were recovered from the dishes by scraping. Lipids were isolated, separated by thin layer chromatography, deacylated, and quantitated on a high performance liquid chromatography system with a Whatman Partisphere SAX column as described (6, 14). We verified that activation of PI 3-kinase was not due to trace amounts of thrombin that persist in the absence of the bacterial protein. Uncut InlB protein that had not been treated with thrombin also stimulated increases in PtdIns(3,4)P2 and PtdIns(3,4,5)P3 in Vero cells, indicating that the protein was active in the absence of thrombin treatment. In addition, 300 ng/ml amounts of the same (cut) preparation of InlB used for the experiments in Fig. 1. Failed to induce shape changes or aggregation in platelets. (Platelets are extremely sensitive to thrombin and aggregate in response to even trace amounts of this protease).

**Infection of Cell Monolayers with Bacterial Strains**— Cultures of wt *L. monocytogenes* strain EGD (BUG600) or the *ΔinlB* isogenic derivative (BUG1047) were grown in brain heart infusion broth (Difco) at 37 °C to an OD600 of 0.80–1.0. Aliquots were then taken, washed three times in DMEM, diluted to approximately 10^7 bacteria/ml, and bacteria were added to cell monolayers at a multiplicity of infection of about 50:1 (bacteria:mammalian cell). The *Salmonella typhimurium* strain SL1344 was grown and prepared for infection as described (8), except that 0.3 M NaCl was included in the LB medium. SL1344 was added to Vero cells at a multiplicity of infection of about 20:1. Contact between *L. monocytogenes* or *S. typhimurium* and cell monolayers was initiated by an overnight incubation (1 200 × g) internalization was allowed to proceed for the indicated times, and cells were then taken for processing for immunoprecipitation or immunofluorescence.

**Immunoprecipitation and Western Blotting**—Approximately 8 × 10^5 Vero cells were seeded in 75-cm^2 tissue culture flasks or 10-cm plates and grown for 40 h. Cells were then starved for 5 h in serum-free DMEM and treated with InlB or EGF or infected with *L. monocytogenes* strain EGD (BUG600). Cell lysates or supernatants were analyzed by immunoprecipitation or immunofluorescence using an antibody against a peptide in the EGF-R (sc-03) or a monoclonal antibody against the EGF-R (clone RC20). The diluted solutions were incubated with the appropriate antibodies for approximately 12 h, followed by a 1-h incubation with protein A-Sepharose beads, washing, and storage at −20 °C. Proteins present in immunoprecipitates were dissociated by boiling for 2 min in sample buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 3 mM sodium orthovanadate, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin, leupeptin, peptatin, and chymostatin. Preclearance and immunoprecipitations were performed at 4 °C as described (6, 14). After preclearance, protein concentrations of the lysates were determined and equal quantities of total protein were used for immunoprecipitations (typically 0.70 mg total protein per immunoprecipitation). After the incubation and washing steps, immunoprecipitates were denatured by boiling in sample buffer for 5 min, and then stored at −20 °C until analysis by SDS-polyacrylamide gel electrophoresis. "Re-immunoprecipitation" experiments were performed as described (15) with some modification. After immunoprecipitation with anti-p85 antibodies, immune complexes were dissociated by boiling for 2 min in sample buffer containing 50 mM Tris-HCl (pH 7.5), 0.5% SDS, and 5 mM dithiothreitol. Protein A-Sepharose beads were then removed by centrifugation, and the supernatants were then incubated with glutathione-activated 30 μl of the resin was applied to a 1-ml column and washed with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 3 mM sodium orthovanadate, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin, leupeptin, peptatin, and chymostatin. The diluted solutions were incubated with the appropriate antibodies for approximately 12 h, followed by a 1-h incubation with protein A-Sepharose beads, washing, and storage at −20 °C. Proteins present in immunoprecipitates were analyzed with an agarose resin coupled to the protease inhibitor p-amino benzenesulfonamide (Sigma, A7555), and InlB preparations were stored as 1–2 mg/ml solutions in 10-μl aliquots at −80 °C. Aliquots were thawed, diluted to 1000-fold concentrated stock solutions in DMEM, and used immediately for experiments involving cell stimulation. Un-
separated on 9.0% (Shc immunoprecipitations) or 7.5% (all other immunoprecipitations) SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Hybond C, Amersham Pharmacia Biotech) with a semidyey apparatus. Membranes were blocked overnight by incubation in Tris-buffered saline (TBS) with 0.1% Tween 20 containing 3% bovine serum albumin (for immunoblotting with anti-Tyr(P) antibodies) or 5% non-fat milk (for all other antibodies). For the anti-Tyr(P) blots, membranes were simply incubated for 1 h at room temperature in TBS-Tween containing anti-Tyr(P) antibodies directly coupled to horseradish peroxidase (RC20-HRPO). For all other blots, membranes were incubated for 1 h in blocking buffer with primary antibody, followed by washing and a second 1 h incubation in blocking buffer containing a protein A-horseradish peroxidase conjugate. After extensive washing of the membranes, proteins were detected by using the ECL or ECL Plus chemiluminescent systems (Amersham) and exposure to film (Hyperfilm MP, Amersham Pharmacia Biotech).

**Results**

**InlB Stimulates PI 3-Kinase Activity in Mammalian Cells—** Infection of Vero cells with a bacterial strain deleted for the *inlB* gene (∆*inlB*) results in smaller increases in PtdIns(3,4,5)P3 than is observed upon infection with wt (∆*inlB*) *L. monocytogenes*, indicating that InlB is needed for full activation of p85-p110 (6). However, the fact that low-level accumulation of PtdIns(3,4,5)P3 is detectable in cells infected with the ∆*inlB* mutant suggests that bacterial factors in addition to InlB contribute to stimulation of p85-p110. On the basis of these results, it was not known whether InlB itself is an agonist of p85-p110 or whether this bacterial protein only plays an auxiliary role in PI 3-kinase activation.

In order to determine whether InlB is sufficient to activate p85-p110, we tested a recombinant InlB protein (Fig. 1A) for stimulation of mammalian PI 3-kinase activity. Treatment of Vero cells for 1 min with soluble InlB at 4.5 nM (300 ng/ml) (Fig. 1A) caused by EGF (Fig. 1B), a known agonist of p85-p110 in some cultured cells (17). Both the InlB- and EGF-induced accumulation of PtdIns(3,4,5)P2 and PtdIns(3,4,5)P3 were transient, being maximal at the earliest time point examined (1 min) and declining thereafter. Accumulation of PtdIns(3,4,5)P2 and PtdIns(3,4,5)P3 in Vero cells in response to EGF is blocked by pretreatment of cells with the PI 3-kinase inhibitor wortman...
nin (19). Similarly, treatment of Vero cells with wortmannin (50 nM) caused a 100 ± 0% (mean ± S.D. of two experiments) and a 96 ± 4% (three experiments) inhibition in the InlB-induced increases of PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃. These results indicate that InlB is an agonist of PI 3-kinase and that activation of p85-p110 by this bacterial protein shares some similarities with activation by EGF.

**InlB Induces Interaction between p85 and One or More Tyrosine-phosphorylated Protein(s)**—Stimulation of p85-p110 by many agonists, including EGF, is accompanied by the interaction of p85 with tyrosine-phosphorylated proteins. Binding of p85 to such proteins, which are often membrane-associated, may allow recruitment of p85-p110 to the plasma membrane and access to its membrane-bound substrates (10).

We had demonstrated previously that infection with *L. monocytogenes* induces the association of p85 with one or more tyrosine-phosphorylated protein(s) in the mammalian cell. This association partly depends on *inlB* and is concomitant with bacterial-induced accumulation of PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ (6). Similarly, treatment of Vero cells with purified InlB induced the appearance of p85 in anti-phosphotyrosine (Tyr(P)) immunoprecipitates (Figs. 2 and 3A), but did not cause detectable tyrosine phosphorylation of p85 itself (Fig. 3B). These results indicate that InlB stimulates an interaction between p85 and tyrosine-phosphorylated protein(s). Stimulation was optimal at about 0.75 nM (50 ng/ml) or higher concentrations (Fig. 2A). Association of p85 with phosphoprotein(s) was maximal at the earliest time point examined (1 min) and declined thereafter (Fig. 2B). These kinetics correlated well with the kinetics of accumulation of PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ in InlB-treated cells. Taken together, these results suggest that tyrosine phosphorylation is involved in PI 3-kinase-mediated signaling induced by InlB.

**InlB Stimulates Tyrosine Phosphorylation of the Adaptor Proteins Gab1, Chl, and Shc and Interaction of These Proteins with p85**—In order to identify the tyrosine-phosphorylated proteins that interact with p85, we immunoprecipitated p85 from cells treated with InlB and detected tyrosine-phosphorylated proteins by Western blotting with anti-Tyr(P) antibodies. Bands corresponding to proteins of approximately 110–120 kDa were associated with p85 in lysates from InlB-treated cells (Fig. 3B, lane 2). We also detected a less prominent band corresponding to a protein of approximately 52 kDa. These 110–120- and 52-kDa phosphoproteins were barely detectable or absent in anti-p85 immunoprecipitates prepared from untreated cells (Fig. 3B, lane 1). Incubation of cells with equivalent molar amounts of the invasion protein InLA (8) or a recombinant (6 × His-tagged) form of the *L. monocytogenes* transcription factor PrfA (18) failed to induce association of tyrosine-phosphorylated proteins with p85, demonstrating that the effects of InlB were relatively specific (data not shown).

We tested if the 110–120-kDa phosphoproteins were focal adhesion kinase (FAK), Gab1, or Chl, proteins known to interact with p85 upon stimulation with certain agonists (14, 17, 21–24). Treatment of Vero cells with InlB stimulated tyrosine phosphorylation of Gab1 and Chl (Fig. 3, C and D), but did not cause increased tyrosine phosphorylation of FAK.2 Treatment with InlB also caused increases in the amount of p85 found in anti-Gab1 and anti-Chl immunoprecipitates (Fig. 3, C and D), but not in anti-FAK immunoprecipitates. We tested if the 52-kDa tyrosine-phosphorylated protein associated with p85 was an isoform of the adaptor protein Shc, which is often present as 48-, 52-, and 66-kDa polypeptides derived from differential translational initiation or alternative splicing (25). Purified InlB caused an increase in tyrosine phosphorylation of the three Shc isoforms in Vero cells and an increase in levels of p85 in anti-Shc immunoprecipitates (Fig. 3E). These results indicate that InlB stimulates tyrosine phosphorylation of the adaptor proteins Gab1, Chl, and Shc and interaction of these proteins with p85. At present, it is not clear if these three adaptors form a single complex with p85 or if multiple complexes containing one or more adaptors and p85 exist.

Reciprocal experiments in which anti-p85 immunoprecipitates were blotted with anti-Shc antibodies confirmed the interaction between Shc and p85 in InlB-treated Vero cells (data not shown). In order to demonstrate the presence of tyrosine-phosphorylated Gab1 in anti-p85 immunoprecipitates, we performed re-immunoprecipitation experiments. Protein com-

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**Fig. 3.** InlB stimulates tyrosine phosphorylation of Gab1, Chl, and Shc and interaction of these proteins with p85. Vero cells were incubated with 3 nM InlB for 1 min (lane 2), incubated with 17 nM (100 ng/ml) EGF for 1 min (lane 3), or left untreated (lane 1), followed by solubilization and immunoprecipitation (IP) with the indicated antibodies. Association of p85 with tyrosine-phosphorylated proteins was analyzed by probing anti-Tyr(P) immunoprecipitates with anti-p85 antibodies (A, lower panel) and by immunoblotting anti-p85 immunoprecipitates with anti-Tyr(P) antibodies (B, upper panel). The arrowheads in B indicate the ~110-kDa phosphoprotein(s) that co-immunoprecipitated with p85, and the small arrow shows the ~52-kDa protein that may be a Shc protein isoform (~66 kDa) or Cbl (~52 and 66 kDa) in E are also indicated. Small arrows highlight ~46- and 52-kDa phosphoproteins in anti-Gab1 immunoprecipitates, which may be Shc proteins. The ~170-kDa phosphoprotein observed in lane 3 of A–E is likely to be the EGF-R. In lane 2 of A, a weak signal corresponding to an unknown tyrosine-phosphorylated protein of ~130–140 kDa is detected from lysates of InlB-treated cells. This phosphoprotein was evident in some but not all experiments involving treatment with InlB (e.g. it is not obvious in Fig. 6A, lane 4), possibly because of interference from the strong Tyr(P) signals of proteins at 110–120 kDa. The second panels in A–E, the membranes were stripped and p85 was detected by blotting with anti-p85 antibodies. For anti-Chl and anti-Shc immunoprecipitations, the membranes were stripped a second time and probed with anti-Chl or anti-Shc antibodies (lower panels) to verify that equivalent amounts of Chl or Shc were in the immunoprecipitates loaded in the different lanes. Similar third blots with anti-Gab1 antibodies were not routinely performed on anti-Gab1 immunoprecipitates, since the antibody often failed to interact with ~110 kDa Gab1 after two rounds of membrane stripping. However, similar experiments in which anti-Gab1 immunoprecipitates were directly probed with anti-Gab1 antibodies showed that InlB treatment did not result in an increase of immunoprecipitable Gab1. Controls with preimmune rabbit IgG demonstrated that the tyrosine phosphorylation of proteins and co-immunoprecipitation of p85 in A–E were specific for the Gab1, Chl, and Shc antibodies. The results in A–E are representative of at least three experiments.
anti-p85 antibodies. Proteins co-immunoprecipitated with p85 were identified by blotting with anti-phosphotyrosine antibodies. Anti-p85 immunoprecipitates that had been re-immunoprecipitated with antibodies against Gab1 (lanes 3 and 5), Cbl (lanes 4 and 6), or with control preimmune rabbit IgG (lane 7). Tyrosine-phosphorylated proteins in the final immunoprecipitates were detected with anti-phosphotyrosine antibodies. Anti-p85 immunoprecipitates that had been re-immunoprecipitated with anti-Gab1 antibodies (lane 5) contained a tyrosine-phosphorylated protein of ~110 kDa with a mobility equivalent to that of the major phosphoprotein in anti-p85 immunoprecipitates that had not been subject to re-immunoprecipitation (lane 2) (all of the lanes in Fig. 4 are from the same SDS-polyacrylamide gel). Similar results were obtained in two other experiments.

The experiments in Fig. 3 showed that, in the absence of bacteria, purified InlB induces tyrosine phosphorylation of Gab1 during infection with L. monocytogenes. In addition to Gab1, Cbl, and Shc, it was important to determine whether InlB controls phosphorylation of these adaptors proteins during bacterial entry into mammalian cells.

A kinetic study indicated that p85 appeared in anti-Tyr(P) immunoprecipitates approximately 5–10 min after infection with L. monocytogenes (Fig. 6A) and remained stable in amount for at least 50 min thereafter. Under these conditions, bacterial entry is not synchronous and occurs at a relatively constant rate from 10 to 90 min after infection. Based on these kinetics, we chose 10 min as the time point of infection at which to examine tyrosine phosphorylation of Gab1, Shc, and Cbl. As expected, infection of Vero cells with wt L. monocytogenes resulted in increased tyrosine phosphorylation of Gab1, Cbl, and Shc and association of these proteins with p85 (Fig. 6, B–D). The effect caused by bacteria was weaker than that provoked by treatment of cells with saturating amounts of InlB protein. In contrast, infection with the &Delta;inlB mutant resulted in almost no induction in tyrosine phosphorylation of Gab1 or association of this protein with p85 (Fig. 6B). Somewhat unexpectedly, infection with the &Delta;inlB strain caused tyrosine phosphorylation of Cbl and Shc about as efficiently as did the wt strain (Fig. 6, C and D). Taken together, these results indicate that endogenous InlB promotes the tyrosine phosphorylation of at least Gab1 during infection with L. monocytogenes. In addition to InlB, L. monocytogenes must produce at least one other factor that contributes to phosphorylation of Cbl and Shc. This unidentified factor may be responsible for the low level but significant activation of PI 3-kinase that occurs upon infection of Vero cells with the &Delta;inlB mutant (6) (see “Discussion”).

**InlB Stimulates PI 3-Kinase-dependent Membrane Ruffling**—Many agonists of PI 3-kinase provoke large changes in the actin cytoskeleton known as membrane ruffling (27–29). Treatment of Vero cells with soluble InlB (3 nM) caused ruffling that was most apparent 5–10 min after treatment and greatly diminished after 30–60 min. Incubation with InlB for 5 min resulted in approximately 75% of the cell population having one or more actin-rich ruffle (Fig. 7B), whereas cells that were not incubated with InlB (Fig. 7A) had essentially no detectable ruffling (ruffles were observed in less than 1% of the cells in the population). InlB-induced ruffling was PI 3-kinase-dependent,
since it was inhibited by treatment of cells with wortmannin (Fig. 7C). As expected, incubation of Vero cells with EGF induced membrane ruffling that was sensitive to wortmannin or AG1478, whereas InlB-mediated ruffling was not sensitive to AG1478 (data not shown). Incubation of cells with wortmannin did not cause a general paralysis of cytoskeletal responses, since “ruffle-like” changes in F-actin provoked by the bacterial pathogen S. typhimurium were not blocked by this treatment (Fig. 7E). Taken together, these results indicate that soluble InlB stimulates changes in the actin cytoskeleton through a mechanism dependent on p85-p110.

_L. monocytogenes_ Does Not Induce Membrane Ruffling during InlB-mediated Entry—Entry of _L. monocytogenes_ into Vero cells and other mammalian cells requires re-organization of the actin cytoskeleton, since uptake is impaired by treatment of cells with cytochalasin D (3, 6). The fact that soluble InlB causes PI 3-kinase-dependent changes in the actin cytoskeleton led us to examine whether we could detect similar changes during uptake of _L. monocytogenes_. Interestingly, infection of Vero cells with wt _L. monocytogenes_ did not result in membrane ruffling (Fig. 7F). In fact, we were unable to detect any obvious accumulation in F-actin near adherent bacteria. We confirmed that bacterial entry was occurring in these conditions at a relatively constant rate from 10 to 90 min after infection by measuring bacterial internalization in parallel with a commonly used antibody labeling technique (12). We also verified that bacterial infection does not somehow inhibit ruffling induced by InlB. Cells were infected with _L. monocytogenes_ for 10 min and then treated with purified InlB for 5 min, resulting in the formation of membrane ruffles (data not shown). Taken together, these results suggest that changes in the actin cytoskeleton that accompany InlB-mediated entry are too small in scale and/or transient to permit detection by laser scanning confocal microscopy. InlB present on the surface of _L. monocytogenes_ appears to cause different cytoskeletal changes than those promoted by InlB that is freely diffusible in solution.

**DISCUSSION**

Our results establish that the _L. monocytogenes_ protein InlB is an agonist of mammalian p85-p110. To our knowledge, InlB is the first reported non-mammalian polypeptide activator of this PI 3-kinase, which is known to be regulated by several growth factors or hormones, including insulin, platelet-derived growth factor, N-formyl-methionyl-leucyl-phenylalanine, thrombin, and EGF. InlB-mediated activation of p85-p110 has features in common with that promoted by these known agonists. Like EGF and insulin, InlB affects p85-p110 in part through tyrosine phosphorylation of adaptor proteins and interaction of these proteins with p85. In fact, InlB regulates phosphorylation of some of the same adaptor proteins utilized by EGF and insulin, including Gab1, Cbl, and Shc (17, 22, 24–26). InlB also resembles these growth factors in its ability to stimulate membrane ruffling through p85-p110.

It is clear that InlB is not the only _L. monocytogenes_ factor that regulates mammalian PI 3-kinase activity. Infection of Vero cells with the ΔinlB mutant results in approximately 30% of the increase in cellular PtdIns(3,4,5)P3 that normally occurs upon infection with wt _L. monocytogenes_ (6). Thus, the ΔinlB mutant is only partly defective in stimulating host PI 3-kinase activity. In this work, we show that the ΔinlB mutant is partly defective in inducing tyrosine phosphorylation of Gab1, but is still able to cause phosphorylation of the adaptors Cbl and Shc. It seems likely that this tyrosine phosphorylation of Cbl and Shc is responsible for the residual activation of p85-p110 caused by the ΔinlB strain. Although the bacterial factor responsible for residual stimulation of PI 3-kinase is not known, it appears to be relatively specific to _L. monocytogenes_. Infection with the non-pathogenic _Listeria_ species _Listeria innocua_ did not result in tyrosine phosphorylation of Gab, Cbl, or Shc or interaction of these proteins with p85. The invasion protein InIA was not responsible for the tyrosine phosphorylation of Cbl and Shc caused by the ΔinlB mutant, since an _L. monocytogenes_ mutant strain deleted for both the inIA and inlB genes (4) was fully capable of inducing phosphorylation of these two adaptors.² Further work is needed to identify the bacterial and mammalian components involved in the residual stimulation of PI 3-kinase.

The sequence determinants in InlB that promote activation of p85-p110 and bacterial entry remain to be defined. InlB contains an NH2-terminal region with eight 22-amino acid repeats that are rich in leucine residues (4, 13, 30), making it a member of the functionally diverse leucine-rich repeat family of proteins (31). We are currently testing whether the leucine-rich repeat region of InlB is sufficient for activation of mammalian PI 3-kinase and bacterial uptake.

The primary recognized function of InlB is in mediating entry of _L. monocytogenes_ into non-phagocytic mammalian cells (4, 5, 12, 34). Our previous results indicated that InlB-mediated entry and activation of p85-p110 involve interaction of p85 with one or more unknown tyrosine-phosphorylated host protein(s) (6). In this report, we identify Gab1, Cbl, and Shc as at least some of the phosphoproteins that are likely to be important for bacterial uptake mediated by PI 3-kinase. Gab1, Cbl, and Shc lack kinase or other known activities and are believed to act as cytosolic “adaptors” that couple p85-p110 and other signaling proteins to surface receptors engaged by a variety of cytokines or growth factors (35). Of these three adaptors, Gab1 may play the most important role in signaling during InlB-mediated entry. The ΔinlB bacterial mutant, which is defective for entry into Vero cells, is also defective in promoting tyrosine phosphorylation of Gab1, but is still able to promote phosphorylation of Cbl and Shc. Although these results are consistent with the idea that a complex composed of phosphorylated Gab1 and p85 is needed for entry of _L. monocytogenes_, definitive evidence for the role of Gab1 or the other adaptor proteins will require genetic approaches that specifi-
cally interfere with the function of each individual protein. Formation of Gabz p85 and Cblz p85 complexes in response to EGF or other agonists is driven by interaction of the SH2 domains in p85 with SH2-binding sites of the type YxxM present in Gab1 and Cbl (17, 22, 24, 36). It seems likely that Gab1z p85 and Cblz p85 complexes induced by InlB also involve such interactions. Shc lacks YxxM sites and is therefore unlikely to directly interact with the p85 SH2 domains. However, the formation of Shcz p85 complexes in response to InlB could involve interaction of Shc with a YxxM-containing protein that is itself associated with p85 (perhaps Gab1 or Cbl) and/or a previously described direct interaction between Shc and the p85 SH3 domain (37).

How do Gab1, Cbl, or Shc form complexes with p85 in response to interaction of the host cell with InlB? We hypothesize that InlB engages a receptor on the surface of the mammalian cell, an idea supported by the fact that purified InlB binds to intact Vero cells or other cell lines (12, 34). Our current model is that binding of InlB to this receptor leads to tyrosine phosphorylation of Gab1, Cbl, and Shc and association of these adaptor proteins with p85. Complexes containing Gab1, Cbl, or Shc and p85-p110 may be recruited to a cytoplasmic region in the InlB receptor, resulting in membrane translocation of p85-p110 and access to its membrane-bound substrate, PtdIns(4,5)P2.

The identity of the putative InlB receptor is not known. Despite some similarities with EGF-mediated signaling, signaling promoted by InlB does not require tyrosine phosphorylation of the EGF-R. InlB-mediated signaling may also be independent of other known receptor tyrosine kinases, such as the insulin, insulin-like growth factor-1, or platelet-derived growth factor receptor, since we do not detect tyrosine-phosphorylated proteins of the expected sizes (95 or 180 kDa) in anti-Tyr(P) or anti-p85 immunoprecipitates from InlB-treated cells (see Fig. 3, A and B). It is possible that the InlB receptor lacks intrinsic kinase activity and is coupled to a cytosolic tyrosine kinase. Clearly, further work is needed to identify the receptor(s) and tyrosine kinase(s) involved in InlB-mediated entry.

How does activation of PI 3-kinase lead to bacterial uptake? Experiments with cytochalasin D indicate that an intact actin cytoskeleton is needed for internalization of L. monocytogenes and most other bacterial pathogens (reviewed in Refs. 1, 3, and 38). Interestingly, purified InlB elicits PI 3-kinase-dependent changes in the actin cytoskeleton (membrane ruffling), raising the possibility that p85-p110 may be involved in cytoskeletal changes that mediate uptake. However, we were unable to detect ruffling or other types of accumulation of F-actin near adherent bacteria. Therefore, the potential role of PI 3-kinase in cytoskeletal changes during entry of L. monocytogenes could not be tested. It is not clear why InlB stimulates membrane ruffling when presented to cells as a diffusible ligand, but not when associated with L. monocytogenes. It is possible that the
gloss cytoskeletal changes caused by soluble InIβ are due to ligand diffusion and engagement of receptors over a large surface, whereas InIβ present on bacteria clusters a more limited number of receptors and induces more local changes that are less readily detectable. Alternatively, the lack of ruffling seen with L. monocytogenes could be due to an insufficient quantity of InIβ interacting with the mammalian cell surface.

Understanding how p85-p110 mediates internalization of L. monocytogenes will require the identification of proteins that act downstream of this lipid kinase in bacterial entry. Several proteins have been demonstrated recently to bind and respond to the lipid products of PI 3-kinase, including the phospholipase PLC-g, some atypical isoforms of protein kinase C, the serine-threonine kinases PDK and Akt, the integrin-binding protein Grp1, and the Arf exchange factor ARNO (11). Another signaling protein that interacts with PtdIns(3,4)P2 and PtdIns(3,4,5)P3 is the GTPase Rac (19). Rac acts downstream of p85-p110 to mediate membrane ruffling in Swiss 3T3 fibroblasts (39) and is also required for ruffling and Fc-γ receptor-mediated phagocytosis in macrophages (40). It will be interesting to determine whether Rac or other GTPases are involved in InIβ-mediated bacterial entry into non-professional phagocytes or in ruffling induced by InIβ.

Acknowledgments—We gratefully acknowledge L. Braun for advice in constructing the InIβ expression plasmid, and L. Braun, H. Gantelet, and P. Steffen for help with protein purification. We also thank R. Hellio for his expertise in confocal microscopy, Prof. H. Chap for his interest and support of this project, and Dr. I. Las a helpful advice. Drs. V. David and R. Hurme are thanked for critically reading the manuscript.

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