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Internalin B Activates Nuclear Factor-κB via Ras, Phosphoinositide 3-Kinase, and Akt*

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Internalin B (InlB), a 630-amino acid protein loosely attached to the surface of *Listeria monocytogenes*, participates in the entry of the bacterium into mammalian cells. This process requires the activation of phosphoinositide (PI) 3-kinase by InlB. Previously, we demonstrated that InlB activates the transcription factor Nuclear Factor-κB in murine J774 macrophage-like cells, an event that also requires PI 3-kinase. Here we have further investigated this phenomenon. InlB activated the small G-protein Ras in J774 cells. Inhibition of Ras with the farnesyltransferase inhibitor manumycin A inhibited NF-κB activation and the recruitment of the p85 subunit of PI 3-kinase, implying that Ras is required for PI 3-kinase activation. InlB also activated the PI 3-kinase downstream effector, Akt, as assessed by increased phosphorylation of Akt on serine 473. Transfection of Hep2 cells with dominant negative Ras N17 or dominant negative Akt inhibited the induction of a reporter gene linked to the interleukin-8 promoter by InlB. Furthermore, the Ras inhibitor manumycin A, the PI 3-kinase inhibitor LY294002, and an Akt inhibitor all blocked the induction of interleukin-8 by InlB. Our study is the first report of a bacterial product activating a pathway involving Ras, PI 3-kinase, and Akt, which leads to NF-κB activation. This process could be involved in host defense or the inhibition of apoptosis during infection.

Listeria monocytogenes is a Gram-positive, food-borne, intracellular bacterium that causes meningitis, septicaemia, abortion, and peritoneal infections. It is capable of infecting both nonprofessional and professional phagocytic cells. The invasion process can be divided into two steps: attachment to the host cell membrane, followed by internalization. Once internalized, the bacterium rapidly lyses the phagocytic vacuole, liberating it into the cytosol. The bacterium quickly starts coating itself with actin filaments, propelling it through the cytosol where it is able to invaginate the plasma membrane of neighboring cell, thus avoiding the immune response (reviewed in Refs. 1–4).

Two proteins, internalin A (InlA)[1] and internalin B (InlB), have been found to be crucial in mediating the internalization of *Listeria* into nonprofessional phagocytes (2, 4). It has been suggested that the different characteristics of each protein determines the efficiency of internalization into specific cell types (4).

InlB is a 630-amino acid protein consisting of eight leucine-rich repeats, each comprising 22 amino acids with high homology with those of InlA, an inter-region containing two repeats of ~70 amino acids, and a 232-amino acid carboxyl-terminal region consisting of tandem repeats each of ~80 amino acids, which begins with the motif GW (5). InlB only loosely associates with the cell surface of *Listeria monocytogenes* via the GW-containing region and appears to be partly released during infection. It has been shown to confer invasiveness to latex beads and noninvasive *Listeria* species when surface-associated (6, 7). The NH_2-terminal leucine-rich repeats are critical for entry (8). Its structure has been recently elucidated (9).

Entry of *L. monocytogenes* is an active process, and part of the mechanism involves activation of phosphoinositide (PI) 3-kinase, because entry is blocked by chemical and genetic inhibition of this enzyme (10). InlB appears to be responsible for PI 3-kinase activation and also induces the recruitment of the mammalian adaptor proteins Shc, Cbl, and Gab-1. Gab-1 in turn has been shown to recruit the p85 subunit of PI 3-kinase (11).

The first receptor described for InlB, gC1q-R, is one of the receptors for the complement protein C1q (12). It does not contain a transmembrane region or glycosylphosphatidylinositol linkage sequence. This would suggest that gC1q-R is acting as a co-receptor for a transmembrane signaling protein capable of initiating signal transduction. A possible candidate has been described by Shen *et al.* (13), who demonstrated that c-Met, the tyrosine kinase receptor for hepatocyte growth factor (HGF), or "scatter factor," can bind InlB. Similar to InlB, HGF has been shown to activate PI 3-kinase, and InlB can induce epithelial cell scattering. Both InlB (14) and HGF (15) also activate the transcription factor NF-κB. For InlB, this may be a host defense response, because NF-κB regulates the expression of many inflammatory genes. Alternatively, NF-κB activation by InlB may be a mechanism whereby *L. monocytogenes* inhibits apoptosis in infected cells, because of the anti-apoptotic effect of NF-κB (16–18).

We have shown that the mechanism of NF-κB activation by

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[1] The abbreviations used are: InlA, internalin A; InlB, internalin B; PI 3-kinase, phosphoinositide 3-kinase; HGF, hepatocyte growth factor; RBD, Raf binding domain; IKK, IκB kinase; NF-κB, nuclear factor κB; FCS, fetal calf serum; GST, glutathione *S*-transferase; PMSF, phenylmethylsulfonyl fluoride; IL, interleukin; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; TK, thymidine kinase.

InlB in J774 macrophages involves PI 3-kinase (14). Here, we have found that, similar to HGF, InlB activates the low molecular weight G-protein Ras. PI 3-kinase activation occurs downstream of Ras and leads to the activation of Akt. Our study therefore identifies signaling processes initiated by InlB, which are involved in NF-κB activation.

EXPERIMENTAL PROCEDURES

Materials—InlB was purified as described previously (7). Anti-pan-Ras antibody was purchased from Oncogene Research Products (Cambridge, MA), whereas the mouse monoclonal antibody, which recognizes the epitope between amino acids 21 and 48 of IκBα, was a kind gift from Prof. Ron Hay (St. Andrews, United Kingdom). Manumycin A (Calbiochem), LY294002 (Sigma), and 1L-6-hydroxymethyl-*chiro*-inositol 2-[(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate] (Alexis Biochemicals) were dissolved in dimethyl sulfoxide. Phospho-Akt and Akt antibodies were obtained from New England Biolabs. Vectors pPL-IL-8-pLuc and pTK-rLuc were kind gifts from Dr. E. Kiss-Toth (University of Sheffield, Sheffield, United Kingdom). The expression vectors encoding dominant negative Ras N17 (described previously; Ref. 19), empty vector Rous sarcoma virus, and the expression vector encoding amino acids 1–149 of human c-Raf1 in pGex-KG, *i.e.* glutathione *S*-transferase (GST)-Ras binding domain (RBD), were kind gifts of Dr. Doreen Cantrell (Imperial Cancer Research Fund, London, United Kingdom). The expression vector encoding dominant negative Akt was donated by Dr. Stephan Ward (Bath University, Bath, United Kingdom). The 22-base pair oligonucleotide, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′, containing the NF-κB consensus sequence (underlined) and T4 polynucleotide kinase kit were from Promega Corp. [γ-^{32}P]ATP (3000 Ci/mol) and the enhanced chemiluminescence ECL reagent were both from Amersham Pharmacia Biotech. Poly(dI-dC) was supplied by Amersham Pharmacia Biotech (Uppsala, Sweden).

Cell Culture—The murine macrophage-like cell line J774 was kindly provided by Prof. Kingston Mills (National University of Ireland, Maynooth, Ireland) and was grown in 10% heat-inactivated fetal calf serum (FCS) (Sigma) in RPMI 1640 (Sigma), which was supplemented with 2 mM L-glutamine (Life Technologies, Inc.), 5% CO_2. The human epithelial cell line Hep2 were grown in 10% FCS, Dulbecco's modified Eagle's medium (Sigma), 2 mM L-glutamine at 5% CO_2.

Ras Activation Assay—J774 cells were cultured at 5×10^6 in 100-mm (10 ml) culture dishes for 24 h in 10% FCS/RPMI 1640, then an additional 24 h in serum-free RPMI 1640, prior to InlB stimulation (500 ng/ml). Cells were washed with ice-cold PBS, and lysates extracted with Ras lysis buffer (50 mM Hepes, pH 7.4, 10 mM NaF, 10 mM iodoacetamide, 75 mM NaCl, 1% Nonidet P-40, 10 mM $MgCl_2$, 1 mM PMSF, 1 mM sodium vanadate, 1 mg/ml β-glycerol phosphate) for 20 min on ice. Equal protein amounts were incubated for 2 h at 4 °C with C-Raf-1 RBD (residues 1–149) precoupled to glutathione-agarose beads (50% slurry). Only activated Ras-GTP will bind to beads, so that activated protein can be pelleted with beads by centrifugation at $2500 \times g$, 3 min, 4 °C. Beads were boiled in SDS-PAGE sample buffer for 5 min and separated on 15% SDS-PAGE. Proteins were transferred to nitrocellulose, immunoblotted with Ras antibody and anti-mouse IgG peroxidase-conjugated antibody, and visualized by chemiluminescence. Equal protein loading was also determined by staining transferred PAGE gel with Coomassie Blue stain.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assays—Assays were conducted as described previously (14); briefly, J774 were seeded at 1×10^5 cells/ml in six-well plates. Relevant concentrations of proteins were added to cells and incubated for 1 h at 37 °C.

Nuclear extracts were prepared by aspiration of media from cells and replacement with ice-cold hypotonic buffer. The subsequent cell pellet was on ice for 10 min. Nuclear-associated proteins were extracted and maintained on ice for 20 min. Following centrifugation, the supernatant was mixed with storage buffer and used immediately, or frozen at −20 °C. Protein concentrations were determined using the method of Bradford (20) and extracts stored at −20 °C.

In the electrophoretic mobility shift assay, nuclear extracts were incubated with 10,000 cpm of a 22-base pair DNA fragment oligonucleotide containing the NF-κB consensus sequence previously labeled with [γ-^{32}P]ATP. Incubation was for 30 min, in the presence of poly(dI-dC) and 10× binding buffer. Incubated mixtures were subjected to electrophoresis on native 5% (w/v) polyacrylamide gels, which were subsequently dried and autoradiographed.

IκBα Immunoblot Analysis—Murine macrophage-like cells J774 were seeded at 5×10^4 cells/ml in six-well plates (3 ml volume), 48 h prior to stimulation and incubated at 37 °C, 5% CO_2. InlB (500 ng/ml) was added, and stimulation was terminated at relevant time points by aspiration of culture media and subsequent addition of 1 ml of ice-cold PBS. After aspiration of PBS from the cells, 100 μl of ice-cold radioimmune precipitation buffer (1% Igepal CA-630, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate in PBS, containing 10 mg of PMSF, 7 μg of aprotinin, and 1 mM sodium vanadate) was added. Plates were shaken on ice for 10 min and cell scraped to ensure lysis. Following further disruption of cells by passage through a 21-gauge needle (10 strokes), an additional 0.1 mg/ml PMSF was added to the samples, which were incubated for 45 min. Samples were then centrifuged for 10 min at $13{,}200 \times g$ at 4 °C, and the supernatant was removed from the cell debris and assayed for protein by Bradford method (20). Equal amounts of protein (4 μg) were resolved by SDS-PAGE and transferred to nitrocellulose, where IκB immunoblot was carried out as described previously (21).

p85 Recruitment Assay—Recruitment assays for p85 were performed as described previously (8). Cells were seeded at 7×10^4 cells/ml in 100-mm culture dishes 48 h prior to stimulation with InlB (500 ng/ml). Inhibitors were preincubated with cells for indicated times where necessary. Cells were washed with cold PBS and then lysed in 1 ml of ice-cold immunoprecipitation buffer for 10 min. Lysates were assayed for protein (20) and precleared with 30 μl of protein A-Sepharose beads (Sigma) for 30 min at 4 °C. The beads were pelleted and the lysates removed. Lysates were incubated with anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology Inc.) for 120 min at 4 °C. The beads were pelleted, the lysate removed, and the beads washed another 3 times. Beads were resuspended in SDS-PAGE sample buffer, boiled for 5 min, centrifuged to remove beads, and then subjected to electrophoresis (7% SDS-PAGE). Proteins were transferred to nitrocellulose, probed with anti-p85α polyclonal antibody, detected with rabbit immunoglobulin G, and visualized with chemiluminescence system (Amersham Pharmacia Biotech).

Akt Immunoblot Analysis—J774 cells were cultured at 5×10^4/ml in 100-mm dishes for 48 h prior to stimulation in serum-free RPMI 1640. Cells were stimulated with 500 ng/ml InlB for the indicated times. Cells were aspirated, washed with ice-cold PBS, and lysed with 100 μl of SDS-PAGE sample buffer. The lysate was sonicated, boiled for 5 min, and centrifuged for 10 min at $13{,}200 \times g$, to remove nuclei and cell debris. Proteins were separated by electrophoresis (SDS-PAGE, 10%), transferred to nitrocellulose, and immunoblotted with antibodies against Phospho-Akt(Ser-473) antibody (New England Biolabs). Secondary antibody rabbit immunoglobulin G was applied and detected by chemiluminescence according to the manufacturer's recommendations (Pierce). Nitrocellulose membranes were stripped of previous antibodies (washed three times with 50 mM glycine, pH 2.0) and reprobed with Akt antibody (New England Biolabs) to determine protein loading.

Transient Transfections—Hep2 cells ($1.5–2 \times 10^4$) were seeded into 96-well tissue culture plates 24 h prior to transfection. Transfections were preformed with SuperFect (Qiagen) according to manufacturer's instructions; each well receiving 500 ng of pPL-IL-8 (which comprises the IL-8 promoter linked to luciferase, which requires NF-κB for its activation) (23), 100 ng of pTK-rLuc for normalization of transfection efficiency, indicated amounts of either dominant negative Akt or Ras N17 for relevant experiments, and pRSV empty vector was used to maintain constant amounts of DNA dose. After transfection (2 h incubation), cells were washed and 100 ml of fresh medium added. Cells were transfected in triplicate for each sample. Twenty-four hours later, cells were pretreated, then stimulated with 500 ng/ml InlB, and incubated for an additional 24 h. Cells were washed with PBS and measured using the dual luciferase system (Promega) as recommended by the manufacturer. Normalized IL-8-promoter-driven activity is the ratio of firefly to *Renilla* luciferase activity.

IL-8 ELISA—Hep2 cells were seeded in 96-well plates (1×10^4 cells in 200 μl) and 24 h later stimulated with 500 ng/ml InlB for 24 h with or without 45 min of pretreatment with inhibitors (manumycin A, LY294002, and Akt inhibitor) diluted in Dulbecco's modified Eagle's medium. Cell supernatants were harvested and assayed for IL-8 by ELISA using the DuoSet ELISA development system for human IL-8 (R&D Systems, Minneapolis, MN), according to manufacturer's instructions.

Statistical Analysis—Significance was evaluated using Student's *t* test for unpaired data.

RESULTS

InlB Induces Activation of the Small G-protein Ras—We have previously shown PI 3-kinase to be involved in InlB-

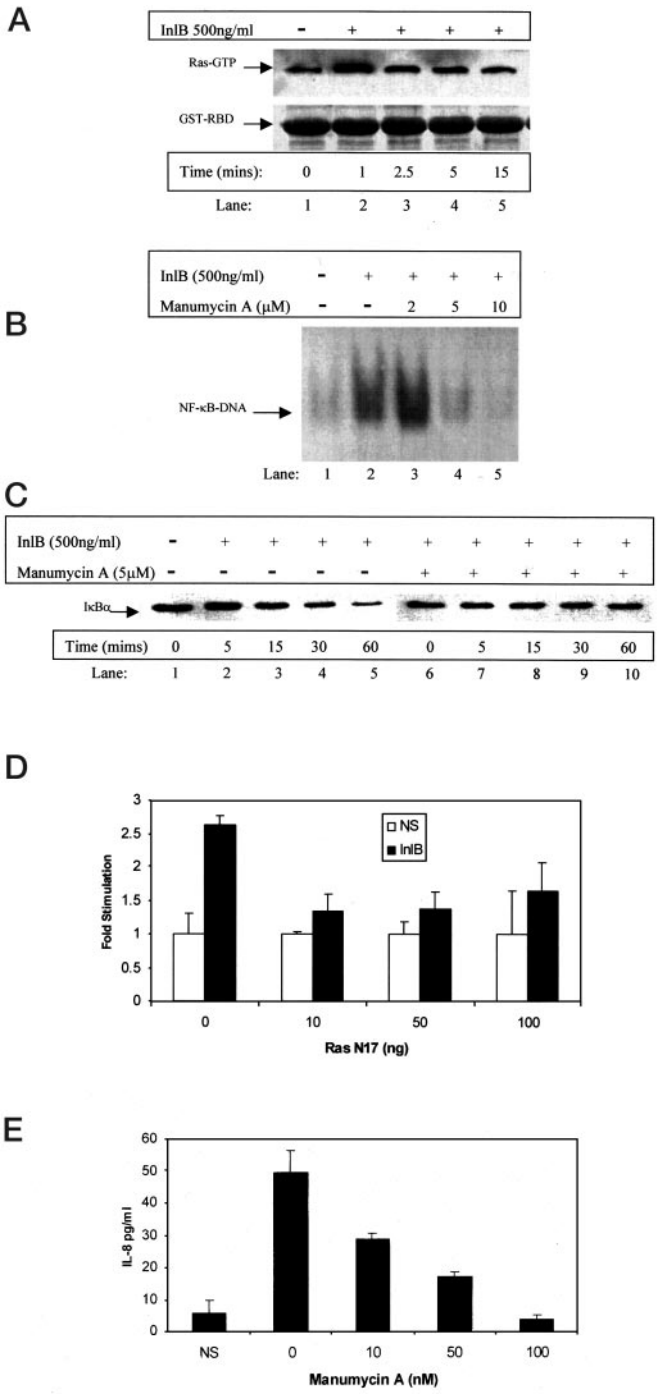

FIG. 1. **InlB induces activation of the small G-protein Ras, which is required for NF-κB activation.** *A*, 5×10^6 J774 cells were grown for 24 h and serum-starved for 24 h prior to stimulation with 500 ng/ml InlB in serum-free media. Activated Ras present in cell lysates was precipitated, as described under "Experimental Procedures." Proteins were separated on 15% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-pan-Ras antibody. Identical results were obtained in two additional experiments. *Lower band* shows GST-RBD and confirms equal protein loading as assayed by Coomassie Blue staining of the proteins remaining on the SDS-PAGE after transfer. *B*, 5×10^4 cells/ml J774 cells were grown for 48 h, pretreated for 60 min with manumycin A (2–10 μM), and stimulated with 200 ng/ml InlB for 60 min. NF-κB activation was determined by electrophoretic mobility shift assay as described under "Experimental Procedures." NF-κB-DNA complexes are shown. Result is representative of three independent experiments. *C*, 5×10^4/ml J774 cells were grown for 48 h, pretreated with manumycin A or media as control for 60 min, and stimulated with 500 ng/ml InlB for 60 min. Equal amounts of protein from cell lysates were separated on 10% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with monoclonal antibody against IκBα. Identical results were obtained in an additional experiment. *D*, Hep2 cells ($1.5–2 \times 10^4$) were transiently transfected with IL-8-luciferase (500 ng), TK-*Renilla*-luciferase, and indicated amounts of Ras N17 for 24 h prior to stimulation with InlB (500 ng/ml, 24 h). Cell extracts were analyzed for luciferase activity. Readings are normalized for each sample as expressed IL-8-luciferase over constitutively expressed TK-*Renilla*-luciferase and plotted as -fold stimulation. Results are mean ± S.D. of triplicate samples and are representative of two additional experiments. *E*, Hep2 cells were seeded at 1×10^4 cells/200 μl in 96-well dishes. After 24 h, cells were pretreated with 0–100 nM manumycin A for 45 min before stimulation with 500 ng/ml InlB. Cells were incubated for another 24 h and cell supernatants collected and analyzed for IL-8 by ELISA. Results are mean ± S.D. of triplicate samples. An identical result was obtained in an additional experiment.

mediated activation of NF-κB (14). We first investigated whether the small G-protein Ras was involved in this response. As shown in Fig. 1*A*, treatment of J774 cells with 500 ng/ml InlB induced an increase in GTP-bound Ras in a time-dependant manner, as assessed by immunoblotting samples for Ras immunoprecipitated from lysates using the Ras-binding domain for Raf, which only recognizes GTP-bound Ras. Maximal activation was detected after 1 min of stimulation (*lane 2*), and the response continued until 5 min after stimulation (*lane 5*). The effect was transient, returning to basal activation levels 15 min after stimulation (*lane 5*).

A role for Ras in mediating InlB-induced NF-κB activation was tested by using the Ras inhibitor manumycin A, which is a potent and selective farnesyltransferase inhibitor (24). Fig. 1*B* illustrates that pretreatment of J774 cells with manumycin A inhibited InlB-mediated NF-κB activation in a dose-dependent manner, with 10 μM abolishing the effect (*lane 5*).

As shown in Fig. 1*C*, manumycin A also prevented InlB-induced degradation of IκBα. InlB induces IκBα degradation from 15 min, most of the IκBα being degraded by 60 min. Manumycin A inhibited this response (compare *lanes 8–10* with *lanes 3–5*). We also tested whether Ras N17, a dominant negative mutant of Ras, could block induction of a reporter gene, luciferase, under the control of the IL-8 promoter, which is NF-κB-dependent. These experiments were carried out in the cell line Hep2, because we found that J774 cells could not be transfected to a high enough efficiency. We have previously shown Hep2 cells to be responsive to InlB in terms of NF-κB activation. InlB induced a 2–2.5-fold increase in luciferase activity. This was the maximum response in these cells (data not shown). Transfection of Hep2 cells with a plasmid encoding Ras N17 inhibited this response, with 50 ng of plasmid abolishing the effect. Because the stimulation of the IL-8 promoter was somewhat weak, and to examine another NF-κB-dependent response, we measured induction of IL-8 by InlB and examined the effect of manumycin A on this response. As shown in Fig. 1*E*, InlB-induced an ~8-fold stimulation of IL-8 expression over nonstimulated cells after 24 h of stimulation. Manumycin A inhibited this effect in a dose-dependent manner, with 100 nM manumycin A abolishing the effect. Taken together, these results imply that Ras is involved in mediating NF-κB activation via InlB.

InlB Induces Recruitment of the p85 Subunit of PI 3-Kinase—Fig. 2*A* demonstrates how the treatment of J774 cells with the PI 3-kinase inhibitor 50 μM LY294002 inhibited the induction of IκBα degradation by InlB (compare *lanes 6* and *7* with *lanes 3* and *4*). As shown in Fig. 2B, Inl*B* induced the rapid recruitment of the p85 subunit of PI 3-kinase to a complex containing tyrosine-phosphorylated proteins in J774 cells. For this, cell lysates prepared from InlB-stimulated J774 cells were immunoprecipitated with an anti-phosphotyrosine antibody, then blotted with anti-p85 polyclonal antibody as described by Braun *et al.* (8). Recruitment was observed 1 min after stimulation (*lane 2*),

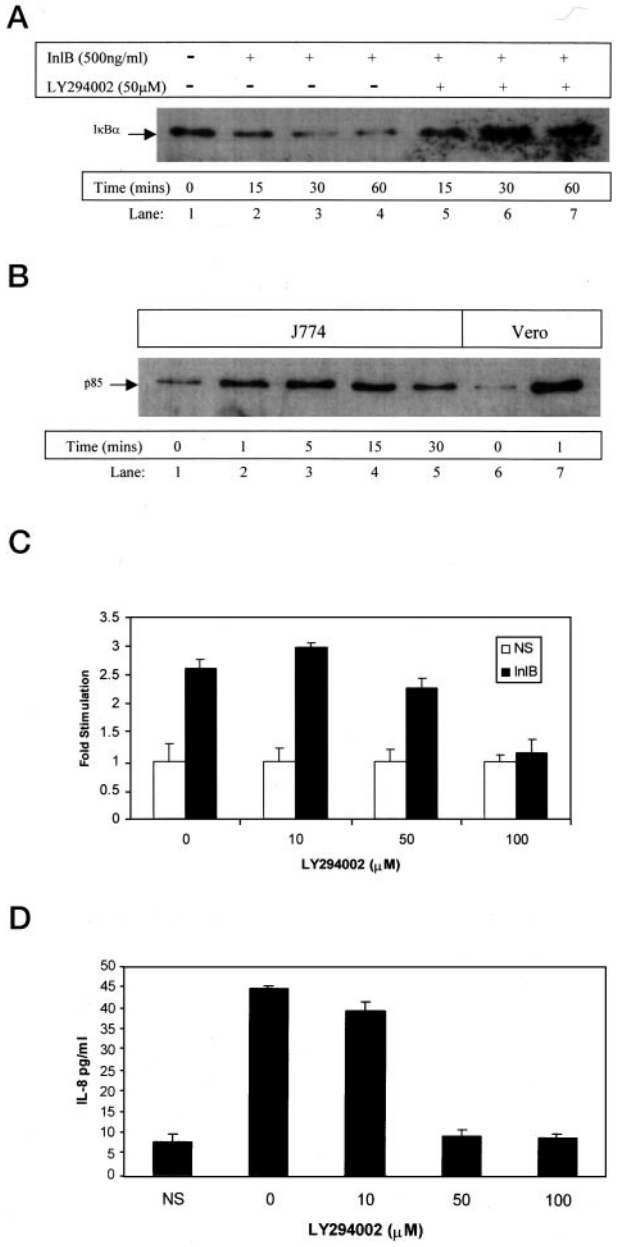

FIG. 2. **PI 3-kinase is involved in InlB-mediated NF-κB activation.** 5×10^4 J774 cells were grown for 48 h, pretreated for 20 min with LY294002 or medium alone, and stimulated with InlB (500 ng/ml) for indicated times. Cells were lysed. Equal amounts of proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose, and immunoblotted for IκBα. Results are representative of three independent experiments. *B*, 7×10^4 J774 or Vero cells were grown for 48 h prior to stimulation with 500 ng/ml InlB for the stated times. Protein extracts from cell lysates were immunoprecipitated with α-phosphotyrosine antibody 4G10, separated by 10% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with polyclonal α-p85 antibody. Results are representative of three independent experiments. *C*, Hep2 cells ($1.5–2.0 \times 10^4$) were transfected with reporter plasmids for IL-8-luciferase and TK-*Renilla*-luciferase for 24 h, pretreated with LY294002, and stimulated with InlB (500 ng/ml, 24 h). Luciferase activity was assayed for each sample. Readings are normalized for each sample as expressed IL-8-luciferase over constitutively expressed TK-*Renilla*-luciferase and plotted as -fold stimulation. Results are means ± S.D. for triplicate determinations. An identical result was obtained in an additional experiment. *D*, Hep2 cells were seeded at 1×10^4 cells/200 μl in 96-well dishes. After 24 h, cells were pretreated with 0–100 μM LY294002 for 20 min before stimulation with 500 ng/ml InlB. Cells were incubated for another 24 h and cell supernatants collected and analyzed for IL-8 by ELISA. Results are mean ± S.D. of triplicate samples. An identical result was obtained in an additional experiment.

reaching a maximum between 5 and 15 min (*lanes 3* and *4*), and began declining at 30 min (*lane 5*). InlB has been shown previously to stimulate p85 recruitment in Vero cells after 1 min, decreasing rapidly thereafter (8, 11). Vero cells were therefore used as a positive control, a sample from cells treated for 1 min with InlB being shown (*lane 7*).

Intriguingly, pretreatment of J774 cells with LY294002, prior to stimulation with InlB, blocked the recruitment of p85 (data not shown). LY294002 is known to act as an inhibitor of the p110 catalytic subunit of PI 3-kinase by covalently binding to the subunit and rendering it catalytically inactive (25). The basis for this inhibition is unclear, but suggests that p85 recruitment depends on p110.

We next investigated whether PI 3-kinase was also involved in NF-κB-dependent transcriptional activity induced by InlB. Again we used the epithelial cell line Hep2. As shown in Fig. 2*C*, InlB induced a 2.5-fold increase in the expression of the luciferase reporter gene. LY294002 inhibited this response, with 100 μM having a maximal effect. To further support this finding, LY294002 was again tested against InlB-induced IL-8 protein expression in Hep2 cells. Fig. 2*D* demonstrates that LY294002 abrogated InlB-induced IL-8 expression in a dose-dependent manner, with 50 μM LY294002 abolishing the effect of InlB.

These results confirm that PI 3-kinase is involved in the activation of NF-κB by InlB.

InlB Mediates Phosphorylation of Akt—We next wished to determine whether InlB was able to activate protein kinase B/Akt, a critical downstream target of PI 3-kinase (26, 27) that has been shown to transiently associate with, and activate, the IκB kinase complex leading to NF-κB activation (28–30). As shown in Fig. 3*A*, InlB induced phosphorylation of Akt as assessed by immunoblotting whole cell lysates with a phosphospecific antibody that recognizes Akt only when phosphorylated on Ser473. Increased phosphorylation was observed within 15 min (*lane 3*) and was optimal 30 min after stimulation (*lane 4*), returning to basal phosphorylation levels at 45 min (*lane 5*). Pretreatment of the cells with the PI 3-kinase inhibitor LY294002, as shown in Fig. 3*B*, blocked the effect (compare *lanes 6–9* with *lanes 2–5*).

We next tested the effect of a plasmid encoding a dominant negative mutant of Akt on activation of the IL-8 promoter. As can be seen in Fig. 3*C*, transient transfection of the dominant negative mutant inhibited the response induced by InlB, with 50–100 ng of plasmid reducing the effect. To further investigate Akt involvement in InlB-mediated NF-κB transcriptional activation, the effect of the recently described Akt inhibitor 1L-6-hydroxymethyl-*chiro*-inositol 2-[(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate] (31, 32) on InlB-mediated IL-8 gene expression was assayed by ELISA in Hep2 cells. As shown in Fig. 3*D*, 1L-6-hydroxymethyl-*chiro*-inositol 2-[(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate] inhibited the expression of IL-8 in a dose-dependent manner, with 10 and 20 μM both having a significant effect, reducing expression by up to 50%. These results indicate that Akt activation is required for NF-κB activation by InlB.

Ras Activation by InlB Occurs Upstream of PI 3-Kinase—We next wished to determine the relationship between Ras and PI 3-kinase in InlB-mediated signaling. J774 cells were pretreated with LY294002 for 20 min, stimulated with 500 ng/ml of InlB, and Ras activation determined at a time range of 0–2.5 min. As can be seen in Fig. 4*A*, LY294002 had no effect on InlB-induced Ras activation. Similar to nontreated cells, Ras activation occurred at 2.5 min of stimulation, which was also evident in LY294002-treated cells.

To further support this, we wished to determine whether the

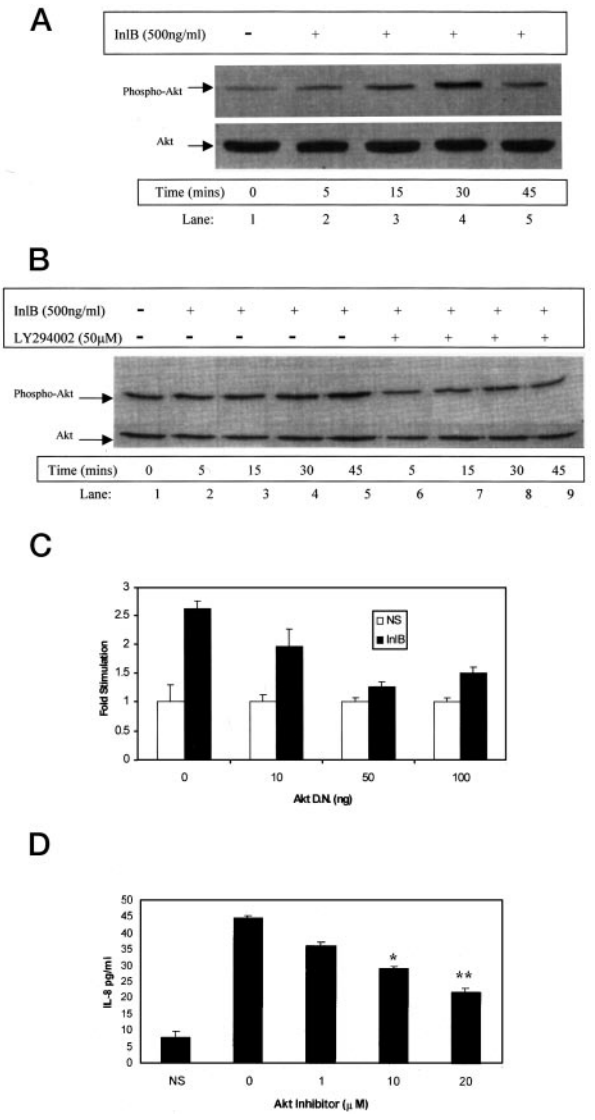

FIG. 3. **Akt is involved in InlB-mediated NF-κB activation.** 7×10^5 J774 cells were grown in serum-free media, 48 h prior to stimulation with 500 ng/ml InlB for indicated times. Protein extracts from cell lysates were separated by 10% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-phospho-Akt antibody (Ser-473). Membranes were stripped and further probed with anti-Akt antibody to determine equal protein loading for each sample. Identical results were obtained from an additional experiment. *B*, 7×10^5 J774 cells were grown for 48 h in serum-free medium, treated or not with 50 μM LY294002 for 20 min prior to InlB (500 ng/ml) stimulation. Proteins extracted from cell lysates were separated by 10% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-phospho-Akt antibody. Membranes were stripped and further immunoblotted for endogenous Akt to determine equal loading. Identical results were obtained from an additional experiment. *C*, Hep2 cells ($1.5–2 \times 10^4$) were transiently transfected with IL-8-luciferase (500 ng), TK-*Renilla*-luciferase, and indicated amounts of dominant negative mutant Akt, 24 h prior to stimulation with InlB (500 ng/ml, 24 h). Extracts were analyzed for luciferase activity. Readings are normalized for each sample as expressed IL-8-luciferase over constitutively expressed TK-*Renilla*-luciferase and plotted as -fold stimulation. Results are mean ± S.D. of triplicate samples. An identical result was obtained in an identical experiment. *D*, Hep2 cells were seeded at 1×10^4 cells/200 μl in 96-well dishes. After 24 h, cells were pretreated with 0–20 μM 1L-6-hydroxymethyl-*chiro*-inositol 2-[(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate] for 30 min before stimulation with 500 ng/ml InlB. Cells were incubated for another 24 h and cell supernatants collected and analyzed for IL-8 by ELISA. Results are mean ± S.D. of triplicate samples. Data indicate significant differences (*, $p = 0.004$; **, $p = 0.005$) when compared with control samples. An identical result was obtained in an additional experiment.

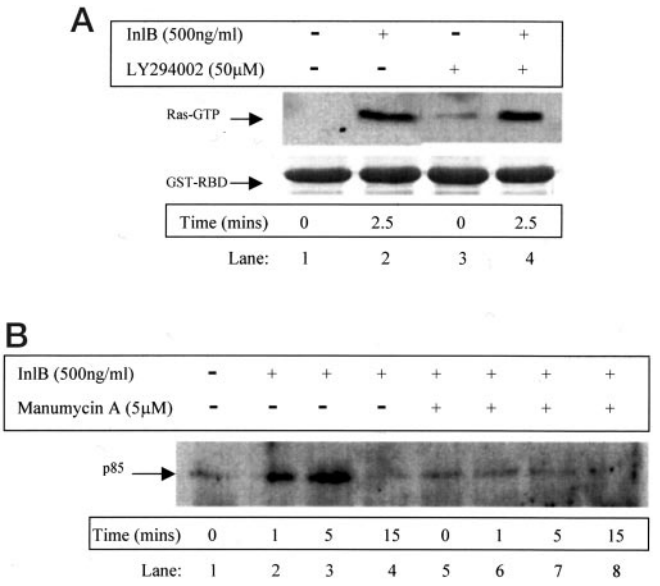

FIG. 4. **InlB-mediated NF-κB activation involves Ras and Akt.** *A*, J774 (5×10^6) cells were grown for 24 h in 10% FCS/RPMI, serum-starved for 24 h, treated for 20 min with serum-free media containing or not containing 50 μM LY294002 prior to InlB stimulation (500 ng/ml). Equal protein amounts from cell lysates were incubated for 2 h with GST-RBD coupled to glutathione-agarose beads. Proteins were separated on 15% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-pan-Ras antibody. *Lower band* shows GST-RBD and confirms equal protein loading as assayed by Coomassie Blue staining of the proteins remaining on the SDS-PAGE after transfer. Identical results were obtained in an additional experiment. *B*, 7×10^5 J774 cells were grown for 48 h, treated with manumycin A for 60 min prior to stimulation with 500 ng/ml InlB. Proteins from cell lysates were immunoprecipitated with α-phosphotyrosine antibody 4G10, separated on 7% SDS-PAGE, transferred to nitrocellulose, and subsequently immunoblotted with α-p85 antibody. Nitrocellulose membranes were stained with Ponceau S to confirm equal loading. Identical results were obtained in an additional experiment.

farnesyltransferase inhibitor manumycin A could block recruitment of p85. J774 cells were pretreated with 5 μM manumycin A for 60 min, stimulated with 500 ng/ml InlB, and then analyzed for p85 recruitment. Fig. 4*B* illustrates that manumycin A was able to totally abrogate the recruitment of p85 to the InlB-mediated phosphorylated complex (compare *lanes 6* and *7* with *lanes 2* and *3*). This strongly suggests that Ras activation occurs upstream of PI 3-kinase in InlB-induced signaling, a conclusion supported by the earlier finding that the timing of Ras activation by InlB precedes recruitment of p85 to the signaling complex.

DISCUSSION

In this study we have analyzed the signaling pathway activated by the *L. monocytogenes* protein InlB, which culminates with the activation of NF-κB. InlB had been shown previously to activate PI 3-kinase (10, 11), a response required for internalization of *L. monocytogenes* (7). Here, we have shown that in J774 macrophages the activation of PI 3-kinase occurs downstream of Ras activation, and that Akt is activated following PI 3-kinase. Our results are the first demonstration of a bacterial protein utilizing Ras, PI 3-kinase, and Akt as signaling mediators that result in NF-κB activation and suggests that PI 3-kinase-mediated internalization may require Ras.

During the course of this research, it was demonstrated that c-Met, the heterodimeric receptor for HGF, was the mammalian receptor for InlB (13, 33). The first reported receptor for InlB was gC1q-R (12), a protein that binds the complement protein C1q. This protein lacks a transmembrane domain and also lacks an identifiable cytoplasmic region. Its role in InlB

signal transduction remains unclear, although it is possible that it interacts with Met. The identification of Met as a receptor for InlB provides a molecular explanation for previously reported InlB signals. These signals include recruitment of Gab-1 and Cbl to phosphotyrosines. The binding of HGF to Met triggers dimerization and autophosphorylation of the receptor, instigating recruitment of adaptor proteins, and initiating several different signaling pathways, including recruitment of Gab-1, Cbl, p85, and Grb-2, this last signal leading to Ras activation (34–36). HGF also activates NF-κB (15), although the post-receptor signals involved are yet to be determined. Our finding that InlB activates NF-κB via Ras and Akt adds two further common signals to the list mediated by InlB and HGF, further strengthening the role of Met in InlB signal transduction.

Our data suggest that Ras is required for PI 3-kinase activation, as indicated by the inhibitory effect of manumycin A on p85 recruitment. Ireton *et al.* (11) have demonstrated that InlB induces the formation of Gab-1.p85 complexes in Vero cells. This may be another means of activating PI 3-kinase. Ras, however, may not play a role in this response in the cells.

It has been suggested that Ras activation of PI 3-kinase is required for optimal response to growth factors, where Ras-GTP activation combined with recruitment of p85 to tyrosine-phosphorylated residues on growth factor receptors synergize to give full activation of PI 3-kinase (37, 38). Our finding that Ras acts upstream of PI 3-kinase, combined with the Ireton *et al.* findings (11) of Gab-1·p85 complex recruitment to InlB-mediated phosphotyrosines, suggests that this interaction could be involved in this response.

Our study also demonstrates that, for Ras, PI 3-kinase and the downstream effector of PI 3-kinase, Akt, are involved in the pathway for NF-κB activation. Akt has been shown to activate the IκB kinase complex by inducing the phosphorylation of IKKα at threonine position 23 (Thr-23) (30), a site claimed to be essential for IKK activation (39). This action causes the phosphorylation of IκB, leading to the release of NF-κB. This pathway may mediate the effect of InlB here. The role of Akt in the activation of the IKK complex is controversial, however, and may be cell type-specific. Akt has been shown to promote transactivation by the p65 subunit of NF-κB (40, 41), thus allowing gene transcription. This process also occurs downstream of Ras. Our data would suggest that Akt might be having a direct effect upon IKK, as inhibition of Ras and PI 3-kinase by specific inhibitors blocked IκBα degradation. A dominant negative mutant of Akt also blocked NF-κB-driven gene transcription, possibly pointing to a role in the pathway of transactivation.

Our evidence that Ras, PI 3-kinase, and Akt are required for NF-κB activation, was supported by data demonstrating that dominant negative Ras N17, LY294002 and dominant negative Akt blocked induction of a reporter gene luciferase, linked to the IL-8 promoter, which is NF-κB-dependent. The stimulation of the promoter by InlB, although weak, was consistently observed, and the signaling inhibitors consistently blocked induction. The basis for the weakness in the response is not clear. To confirm this result, we measured IL-8 protein production. This gave a much stronger response to InlB. We again tested LY294002 in the response and found it to be inhibitory. We were unable to test dominant negative mutants in this assay, because it is not transfection-based, and Hep2 cells are not transfectable to a high enough efficiency to ensure that enough cells in the population would be susceptible to inhibition. Therefore, we again tested the Ras inhibitor manumycin A and a newly described inhibitor of Akt. Both abolished the effect of InlB on IL-8 production, confirming the result obtained in the IL-8 promoter assay, and providing additional support for Ras and Akt as possible signal transducers for InlB.

Activation of NF-κB by InlB may be part of the host defense response, given that we have shown that InlB induces NF-κB-dependent inflammatory cytokine expression. However, InlB may also be promoting an anti-apoptotic response required for survival of *L. monocytogenes*-infected cells. This response may require Akt, which, along with being involved in NF-κB activation, is also involved in phosphorylation and the inactivation of the pro-apoptotic proteins Bad and pro-caspase 9 (22, 42–44). The effect of Akt on NF-κB has been shown to render cells partially resistant to the pro-apoptotic effects of etoposide (39), supporting a role for Akt/NF-κB in any anti-apoptotic response to *L. monocytogenes*.

In conclusion, our results identify Ras and Akt as novel signals activated by InlB, which are required for NF-κB activation, with Ras acting upstream of PI 3-kinase. Whether Ras and Akt are also required for InlB-mediated internalization of *L. monocytogenes* is under investigation, although, given that PI 3-kinase is required for this process, there is a strong possibility that this will be the case. These results concur with Met as the receptor for InlB and elucidate a pathway important for the interaction between this bacterium and the host cell.

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