INFECTION AND IMMUNITY, Apr. 2005, p. 2061–2074 Vol. 73, No. 4
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.4.2061–2074.2005

Redundant Roles for Met Docking Site Tyrosines and the Gab1 Pleckstrin Homology Domain in InlB-Mediated Entry of *Listeria monocytogenes*

Tumay Basar, Yang Shen, and Keith Ireton*

Department of Medical Genetics and Microbiology, University of Toronto, Toronto, Ontario, Canada

Received 13 August 2004/Returned for modification 9 November 2004/Accepted 19 November 2004

The bacterial pathogen *Listeria monocytogenes* causes food-borne illnesses leading to gastroenteritis, meningitis, or abortion. *Listeria* induces its internalization into some mammalian cells through interaction of the bacterial surface protein InlB with host Met receptor tyrosine kinase. Binding of InlB leads to phosphorylation of Met and the adapter Gab1 and to activation of host phosphoinositide (PI) 3-kinase. The mammalian ligand of Met, hepatocyte growth factor, promotes cell motility and morphogenesis in a manner dependent on phosphorylation of two docking site tyrosines at positions 1349 and 1356 in the receptor's cytoplasmic tail. Here we determined if these tyrosines were essential for *Listeria* entry. A derivative of the human cell line T47D stably expressing a truncated Met lacking most of its cytoplasmic domain was unable to support InlB-mediated signaling or entry. Surprisingly, cells expressing mutant Met containing phenylalanine substitutions in both tyrosines 1349 and 1356 (MetYF) allowed entry and InlB-induced Gab1 phosphorylation. However, in contrast to the situation in cells expressing wild-type Met, Gab1 phosphorylation in MetYF cells required PI 3-kinase activity. The Gab1 pleckstrin homology (PH) domain was constitutively associated with the plasma membrane of cells in a PI 3-kinase-dependent manner. Overexpression of the PH domain blocked entry of *Listeria* into cells expressing MetYF but not into cells expressing wild-type Met. Taken together, these results indicate that the docking site tyrosines are dispensable for internalization when membrane localization of Gab1 is constitutive. Distinct pathways of recruitment by phosphorylated tyrosines in Met and PH domain ligands in the membrane are redundant for bacterial entry.

Listeria monocytogenes is a gram-positive, food-borne bacterial pathogen capable of causing gastroenteritis, meningitis, or abortions (43). *Listeria* induces its own internalization (entry) into nonphagocytic mammalian cells, a process that is likely to play an important role in traversal of the intestinal, placental, and blood-brain barrier cells (6, 7, 22). One of the pathways of internalization of *Listeria* is mediated by interaction of the bacterial surface protein InlB with its host receptor, the Met receptor tyrosine kinase (39).

InlB-Met interaction leads to bacterial engulfment through an incompletely understood process that requires activation of the host type IA phosphoinositide (PI) 3-kinase p85/p110 (16, 17, 39) and host signaling events that regulate the actin cytoskeleton (2). *Listeria*-induced activation of p85/p110 coincides with formation of a complex between the p85 regulatory subunit and the tyrosine-phosphorylated host adapter protein Gab1 (17, 39). The function of Gab1-p85 complexes may be to recruit PI 3-kinase to the membrane, allowing access to its major substrate phosphatidylinositol 4,5-bisphosphate, which is subsequently converted to phosphatidylinositol 3,4,5-trisphosphate (PIP3).

Met is a disulfide-linked heterodimer consisting of ~55-kDa α and ~145-kDa β-chains (4, 41). The α subunit is exclusively extracellular, whereas the β-chain consists of extracellular, transmembrane, and cytoplasmic domains. The cytoplasmic region of Met contains a kinase domain and multiple sites of tyrosine phosphorylation, including tyrosines 1234 and 1235 located in the activation loop. When phosphorylated, these two tyrosines promote upregulation of Met kinase activity (3, 41). While tyrosines 1234 and 1235 are the major sites of phosphorylation (10, 11, 48), at least three other tyrosines, located at positions 1003, 1349, and 1356, have been either demonstrated or inferred to be phosphorylated in response to cell stimulation. When phosphorylated, these three tyrosines recruit various downstream signaling proteins. Tyrosine 1003, located in the juxtamembrane region of Met, is needed for recruitment of the amino-terminal tyrosine kinase binding domain of the E3 ubiquitin ligase, Cbl (34). Tyrosines 1349 and 1356 constitute a multisubstrate docking site that associates with the SH2 domains of several signaling molecules, including the adapter Grb2, the tyrosine kinase Src, phospholipase C-γ (PLC-γ), and p85 (11, 19, 35, 48). The docking site tyrosines are also needed for interaction of Gab1 with Met (1, 31). Phospho-Y1349 binds directly to a 16-amino-acid sequence within the Met binding domain (MBD) in Gab1 (24), and phospho-Y1356 indirectly recruits Gab1 through association with Grb2 and Gab1 complexes (23). Studies from several laboratories indicate that tyrosines 1349 and 1356 in Met are essential for hepatocyte growth factor (HGF)-induced scattering and morphogenesis (tubulogenesis) in Madin-Darby canine kidney (MDCK) cells and for proper development of the liver and placenta in mice (11, 25, 36, 48). Tyrosine 1003 appears to play a negative role in Met-mediated transformation of cells (34).

The Gab1 adapter plays a critical role in signaling and biological function downstream of Met (3). Upon membrane re-

* Corresponding author. Mailing address: Department of Medical Genetics and Microbiology, University of Toronto, Toronto, Ontario M5S 1A8, Canada. Phone: (416) 946-5356. Fax: (416) 978-6885. E-mail: k.ireton@utoronto.ca.

cruitment, Gab1 becomes phosphorylated on multiple tyrosine residues that serve as recruitment sites for several SH2 domain-containing proteins including p85, the tyrosine phosphatase Shp2, PLC-γ, and the adapter protein Crk. Docking of Crk or PLC-γ to Gab1 is needed for Met-mediated tubulogenesis (14, 21). Shp2 recruitment to Gab1 plays critical roles in both scattering and morphogenesis (20, 29, 38).

In addition to being recruited to the activated Met receptor, Gab1 exhibits a second mechanism of association with the plasma membrane. This adapter has an amino-terminal pleckstrin homology (PH) domain that binds preferentially to PIP3 (27, 28). The Gab1 PH domain mediates membrane translocation in response to stimulation of cells with serum, HGF, or epidermal growth factor (EGF) (27, 28, 37). PH domain-dependent translocation appears to be promoted by interaction with PIP3, since it is inhibited by treatment of cells with chemical inhibitors of PI 3-kinase.

The dual mechanisms of recruitment of Gab1 to either phosphorylated Met or PIP3 suggest a potential for functional redundancy. In this work, we find that entry of *Listeria* into host mammalian cells is independent of tyrosines 1349 and 1356 in Met, under conditions in which the Gab1 PH domain is constitutively associated with the plasma membrane. When constitutive membrane recruitment of Gab1 is inhibited through overexpression of the isolated PH domain, bacterial internalization becomes dependent on tyrosines 1349 and 1356. The two mechanisms of recruitment of Gab1 are redundant for entry of *Listeria*.

MATERIALS AND METHODS

Bacterial strains, mammalian cell lines, and media. The *L. monocytogenes* Δ*inlA* and Δ*inlAB* mutant strains used are isogenic with the wild-type strain EGD and contain in-frame mutations in the *inlA* and/or *inlB* genes (9). These strains were grown as described (9).

The mammalian cell lines used are the African green monkey kidney cell line Vero (ATTC CRL-1587), the human mammary epithelial cell line T47D (ATTC HTG-133), a derivative of T47D cells (T47D/Metwt) stably expressing wild-type Met receptor (39), and transfected clones of T47D cells, generated in this study, that stably express Met deleted of most (~80%) of the cytoplasmic domain (T47D/MetΔcyto) or Met containing tyrosine-to-phenylalanine substitutions at amino acids 1349 and 1356 (T47D/MetYF). All of these cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g of glucose (catalog no. 11995-065; Gibco-BRL) per liter, 2 mM glutamine, and 10% fetal bovine serum (FBS). During routine expansion and propagation of the T47D cell lines expressing wild-type and mutant Met alleles, 0.4 mg of G418 per ml was included to select for the Met cDNA. When cells were seeded for bacterial infection or stimulation experiments, G418 was not included. Cell growth, cell stimulation, and bacterial infections were all carried out at 37°C in 5% CO_2.

Antibodies, inhibitors, and other reagents. The polyclonal antibodies used were rabbit anti-Gab1 (catalog no. 06-579; Upstate Biotechnology [UBI]), rabbit anti-human Met C-12 (catalog no. sc-10; Santa Cruz Biotechnology), affinity-purified goat anti-Met extracellular domain (catalog no. AF276; R&D Systems), normal goat immunoglobulin G (catalog no. AB-108-C; R&D Systems), rabbit anti-phospho mitogen-activated protein kinase (MAPK; thr202/tyr204) (catalog no. 9101; Cell Signaling Technology), rabbit anti-phospho Akt (serine 473) (catalog no. 9271; Cell Signaling Technology), and rabbit anti-*L. monocytogenes* R11 (12). The monoclonal antibodies used were mouse anti-human Met DL-21 (catalog no. 05-238; UBI), mouse anti-human Met DO-24 (catalog no. 05-237; UBI), anti-phosphotyrosine 4G10, anti-hemagglutinin (HA) (catalog no. MMS-101R; Babco), and anti-tubulin (catalog no. T5168; Sigma). Secondary antibody horseradish peroxidase (HRPO), alkaline phosphatase, Cy5, or fluorescein isothiocyanate (FITC) conjugates were from Jackson Immunolabs. Anti-rabbit Texas Red, anti-mouse BODIPY FL, and Texas Red X-phalloidin were from Molecular Probes (catalog no. T-6391, B-2752, and T-7471, respectively). Recombinant HGF was purchased from Sigma (catalog no. H1404) or R&D systems (catalog no. 294-HGN). The PI 3-kinase inhibitor LY294002 was from Sigma (catalog no. L9908).

Construction of stably transfected cell lines. cDNAs encoding full-length Met containing tyrosine-to-phenylalanine substitutions in both amino acids 1349 and 1356 (MetYF) or Met deleted for most of the cytoplasmic domain (MetΔcyto) were gifts of M. Park (McGill University). MetYF was made by subcloning a fragment containing the mutations in tyrosines 1349 and 1356 from the original colony stimulating factor-Met chimeric background (11) into the cDNA for full-length wild-type Met. The MetΔcyto allele ends at codon 1037 of Met, followed by an in-frame 30-amino-acid sequence containing three tandem HA tags. MetΔcyto encodes a protein lacking the last 352 amino acids of the 435-residue cytoplasmic region, resulting in removal of the entire kinase domain and the docking site tyrosines. Both the MetΔcyto and MetYF cDNAs were subcloned into the amphotropic retroviral expression vector pLXSN. Viral supernatants containing packaged mutant Met plasmids were generated by cotransfection of the pLXSN-Met construct with the helper plasmid pSVψA-MLV into 293T cells, essentially as described (47).

Clones of T47D/MetΔcyto or T47D/MetYF cells were generated by infection of subconfluent T47D cells with viral supernatant overnight in the presence of 4 mg of polybrene per liter. Virus-containing medium was removed, and cells were washed once with DMEM with 10% FBS and glutamine and grown in the same medium for another ~24 h. Infected cells were then trypsinized, adjusted to a concentration of 10^4 cells/ml, and threefold serial dilutions in DMEM with 10% FBS, Glutamax I (35050-061; Gibco), and 0.5 mg of G418 (11811-031; Gibco) per liter were performed in 96-well plates. After about 14 days, single colonies obtained by limiting dilution were trypsinized and expanded in medium with G418.

Approximately 20 clones each of T47D/MetΔcyto and T47D/MetYF cells were screened for Met expression by Western blotting by using anti-Met DL-21 antibody. Quantitative Western blotting was performed by using antibody DL-21, followed by anti-mouse-HRPO, and incubation with ECL Plus (catalog no. RPN2132; Amersham Biosciences). Reaction of HRPO with ECL Plus reagent generated fluorescence at 503 nm, which was detected with a Storm apparatus (Molecular Dynamics). In parallel with each Western blot comparing expression in T47D/Metwt and T47D/MetΔcyto or T47D/MetYF cells, a control was performed to ensure that conditions were within the linear range for detection. Twofold serial dilutions of lysates from T47D/Metwt cells were loaded and detected. ImageQuant software (Molecular Dynamics) was used to quantify relative expression levels. Five clones each of T47D/MetΔcyto and T47D/MetYF cells were identified that express between 50 and 150% of the level of Met as that in our clone of T47D/Metwt cells (39). Each of the T47D/MetΔcyto or T47D/MetYF clones was found to have similar abilities to support bacterial entry. T47D/MetΔcyto clone 2 and T47D/MetYF clone 23, each at early passages expressing ~150% the level of Met in T47D/Metwt cells, were used in subsequent experiments.

The tyrosine-to-phenylalanine substitutions at positions 1349 and 1356 were verified to be present in the chromosome of T47D/MetYF cells (clone 23) by purification of chromosomal DNA and PCR amplification with primers 5′-(CCG)GAATTCATCCTAACTAGTGGGG-3′ and 5′-(CGC)GGATCCGACGGTATCGATAAG-3′ (underlining indicates the EcoRI and BamHI sites, respectively) corresponding to nucleotides 3100 to 3115 in Met and sequences downstream of Met in pLXSN. These primers allowed amplification of the mutant Met cDNA in pLXSN, but not the endogenous Met gene. The resulting PCR products were digested with EcoRI/BamHI, subcloned into pBluescript (Stratagene), and two clones were sequenced. Each clone contained the expected mutations at codons 1349 and 1356 in Met.

Flow cytometry was used to compare surface expression of Met in T47D/MetΔcyto (clone 2) and T47D/MetYF (clone 23) cells to that in T47D/Metwt cells and in parental T47D cells. Adherent cells in six-well plates were incubated on ice with 25 mg of goat anti-Met extracellular domain or control goat immunoglobulin G per liter, followed by incubation with anti-goat-FITC. Cells were recovered by scraping with a rubber policeman, and propidium iodide (PI) was added to a concentration of 1 μg/ml to allow differentiation of live (PI-negative) versus dead (PI-positive) cells. Labeled cells were assayed by using a FACSCalibur instrument (Beckton Dickinson), employing two channels to detect FITC and PI. Data were analyzed by using CellQuest (Beckton Dickinson), and PI-negative cells in the population were assessed for relative levels of FITC fluorescence (Met expression). Based on these experiments, T47D/MetΔcyto (clone 2) and T47D/MetYF (clone 23) cells were found to express levels of surface Met that were about 170 and 140% of that in T47D/Metwt cells, respectively, and about 10-fold higher than that in T47D cells.

Protein expression and purification. Recombinant InlB protein was expressed in *Escherichia coli* and purified essentially as described (17).

Plasmid constructs containing glutathione transferase (GST) fused to the SH2 domain of Grb2 (11), the Gab1 MBD (23), or the carboxyl-terminal SH2 domain of p85 (46) were expressed in *E. coli* strain Top10 after growth in Luria-Bertani broth containing 100 μg of ampicillin per ml to an optical density at 600 nm of ~0.50 and addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside for 3 h. Bacterial cultures were centrifuged at 4°C for 10 min, and the pellet was washed once with ice-cold buffer A (25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA) and then resuspended in 1/20th volume cold buffer B (20 mM HEPES, 120 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μg each of aprotinin and leupeptin). Pellets were lysed by sonication on ice three times for 15 seconds by using output 3 of a Branson Sonifier 450. Lysates were centrifuged at 15,000 rpm for 30 min in a Hereaus picofuge, the supernatants were collected, and Igepal CA-630 (I-3021; Sigma) was added to 0.5%. A total of 20 ml of lysate (corresponding to 400 ml of starting bacterial culture) was incubated with 0.6 ml of a 50% slurry of glutathione Sepharose 4B beads (17-0756-01; Amersham Biosciences) on a rotating wheel for 1 h at 4°C. Beads were washed six times with cold buffer B with 0.5% Igepal CA-630, resuspended in 0.50 ml of the same buffer, and stored at −80°C until use.

Gab1 constructs. HA-tagged wild-type Gab1 (HA-Gab1wt) or Gab1 deleted of the PH domain (HA-Gab1ΔPH) were generously provided by M. Holgado-Madruga and A. Wong (Kimmel Cancer Institute, Philadelphia, Pa.). HA-Gab1wt and HA-Gab1ΔPH alleles were subcloned into the mammalian expression vector pcDNA3.1 (Invitrogen). A construct encoding the PH domain of Gab1 with enhanced green fluorescent protein (EGFP) fused to its carboxyl terminus was made by using primers 5′-(CG)GGATCCATGAGCGGCGGCG AAGTGGTT-3′ and 5′-(CG)GAATTCTCATCCACAGATGTCACAGAT-3′ (underlined sequences indicate BamHI and EcoRI sites, respectively) and pcDNA.HA-Gab1wt as a template. The PCR and a mixture of *Taq* and *Pfu* DNA polymerase were used to amplify a DNA fragment encoding amino acids 1 to 116 containing the PH domain of Gab1. The resulting PCR product was digested with BamHI/EcoRI and subcloned into the BglII/EcoRI sites of EGFPC1 (Clontech). The EGFP-PH construct was verified by DNA sequencing.

Transient transfection of mammalian cells. A total of 6×10^4 Vero or 2×10^5 T47D/Metwt or T47D/MetYF cells were seeded on 22- by 22-mm glass coverslips in six-well plates and grown for ~24 h prior to transfection with EGFPC1, EGFP-PH, pcDNA.HA-Gab1wt, or pcDNA.HA-Gab1ΔPH plasmid DNA. Two micrograms of DNA and 3.5 μl of LF2000 reagent (catalog no. 11668-027; Gibco-BRL) were used per coverslip. In some experiments (see Fig. 9), cotransfection of EGFP-PH and pcDNA.Ha-Gab1wt or pcDNA.Ha-Gab1ΔPH was performed by using 2 μg of each plasmid DNA. Transfections were done in the absence of FBS, and cells were incubated with LF2000-DNA complexes for ~6 (see Fig. 6) or ~16 h (see Fig. 9).

Infection of cell monolayers and measurement of bacterial entry. Infection of mammalian cells with the *L. monocytogenes* strains Δ*inlA* or Δ*inlAB* was as described (17, 39). A multiplicity of infection (MOI) of ~50:1 was used for the entry experiments assayed by gentamicin protection or immunofluorescence microscopy. An MOI of ~150:1 was used in the cell stimulation and precipitation experiments (see Fig. 3,4, 5, and 8). Similar, but quantitatively weaker, results were obtained when an MOI of ~50:1 was used for stimulation. Gentamicin protection assays to measure intracellular bacteria (Fig. 1) were as described (30, 39).

For quantification of bacterial entry by fluorescence microscopy (see Fig. 9), we modified an approach previously used to measure entry of *L. monocytogenes* in individual mammalian cells in a population (5). After transfection with EGFP, EGFP-PH, pcDNA.Ha-Gab1wt, and/or pcDNA.Ha-Gab1ΔPH, cells grown on glass coverslips were washed and incubated in medium with 10% FBS for ~5 h prior to bacterial infection. Host cells were washed in DMEM and incubated with bacterial suspensions in DMEM for 1 h. Nonadherent bacteria were removed by washing with DMEM, and infected cells were incubated for an additional 30 min. Cells were then washed with phosphate-buffered saline (PBS) and fixed in cytoskeleton buffer (17) containing 3% paraformaldehyde. Extracellular bacteria were labeled by 1-h incubation in anti-*Listeria* antibody R11 in Tris-buffered saline with 0.5% bovine serum albumin (BSA), followed by an additional hour in anti-rabbit Texas Red. Mammalian cells were permeabilized by a 5-min incubation in cytoskeleton buffer with 0.4% Triton X-100, followed by 1-h incubations in R11 and then anti-rabbit Cy5. This procedure resulted in extracellular bacteria being labeled with both Texas Red and Cy5, and intracellular bacteria was labeled only with Cy5. A motorized Leica DMIRBE fluorescence microscope equipped with an Orca C4742-95 CCD camera (Hamamatsu), filters for detection of FITC-EGFP, Cy5, and Texas Red, and OpenLab version 2.25 software (Improvisions) were used for analysis of bacterial entry. Briefly, EGFPC1- or EGFP-PH-positive mammalian cells were identified, and images of six to eight serial, optical sections of 1-μm thickness were captured. Optical sections started just above the host cell and ended below the cell. For each section, images of EGFP, Cy5, and Texas Red fluorescence were acquired. Individual serial sections corresponding to Cy5 or Texas Red fluorescence were superimposed so as to create a composite image containing all Cy5- or Texas Red-positive bacteria throughout the host cell. The Cy5 and Texas Red composite images (artificially colored in red and green, respectively) were then overlaid, with the result that extracellular bacteria appear yellow and intracellular bacteria appear red. Finally, the EGFP fluorescent image (artificially colored blue) was superimposed on the Cy5 and Texas Red overlay to allow identification of bacteria associated with transfected cells.

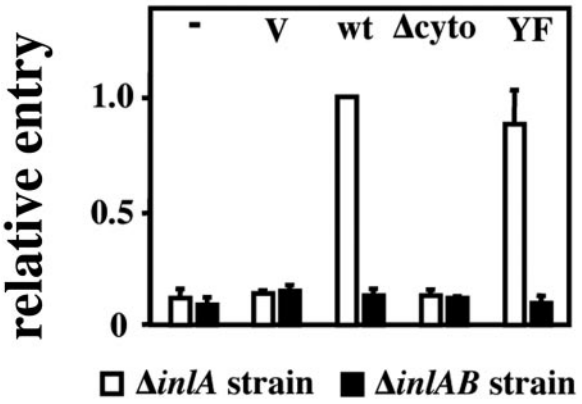

FIG. 1. InlB-dependent entry of *Listeria* into T47D cells requires the cytoplasmic domain of Met but not tyrosines 1349 and 1356. Cells were infected with the Δ*inlA* or Δ*inlAB* mutant strains of *L. monocytogenes*, and a gentamicin protection assay was performed as described in Materials and Methods. −, parental, untransfected T47D cells; V, T47D cells transfected with the empty vector pLXSN; wt, cells stably expressing Metwt; Δcyto, cells expressing MetΔcyto; YF, cells expressing MetYF. The data are presented as the average relative entry values ± standard deviations from three to six independent experiments, depending on the cell line. In each experiment, infection with a given strain was performed in duplicate. Percent entry is the percentage of the initial inoculum that survived gentamicin treatment after a 1-hr infection in the absence of antibiotic, followed by a 2-hr incubation in the presence of gentamicin. To obtain the relative entry values, the absolute percent entry values in each experiment were normalized to that of the Δ*inlA* strain in T47D/Metwt cells.

Immunoprecipitation, GST fusion protein precipitation, and Western blotting. A total of 1.3×10^6 T47D or T47D/Metwt or 1.5×10^6 T47D/MetΔcyto or T47D/MetYF cells were seeded in 10-cm plates and grown for ~40 h before starvation for 5 h in DMEM without FBS. In some experiments, cells were starved for ~16 h in DMEM with 0.1% FBS followed by another 5 h in DMEM without FBS. (The two different starvation conditions yielded similar results). In some experiments, starved cells were incubated in DMEM with 100 μM LY294002 or the vehicle Me_2SO (0.2%) for 45 to 60 min prior to a 5-min treatment with 1.5 nM InlB or 0.5 nM HGF or a 10-min infection with *L. monocytogenes* strains. Cell lysis and immunoprecipitation were as described (39), with the exception that hydrogen peroxide was used to activate sodium orthovanadate for experiments involving analysis of Gab1 phosphorylation in cells infected with bacteria (see Fig. 5B and 8A). Activation of vanadate by this method was found to be necessary to preserve Gab1 phosphorylation in *Listeria*-infected cells. In all other experiments, vanadate was activated by repeated adjusting of the pH to 10, followed by boiling. For precipitation with GST fusion proteins, 1 ml of each cell lysate was precleared for 45 min by incubation with 20 μl of a 50% slurry of glutathione Sepharose 4B beads. Following preclearance, protein concentrations were determined by using a bicinchoninic acid kit (Pierce). Fifteen milliliters of a slurry of glutathione Sepharose beads complexed with the GST fusion protein of interest was added to 0.5 to 1.0 mg of cell lysate and incubated on a rotating wheel at 4°C for 2 h. GST fusion protein and glutathione Sepharose complexes were precipitated by centrifugation, washed twice in lysis buffer (39), washed once in wash buffer (0.2% Triton X-100, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 3 mM sodium orthovanadate, 20 mM NaF, and 1 mM phenylmethylsulfonyl fluoride), denatured by the addition of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sam-

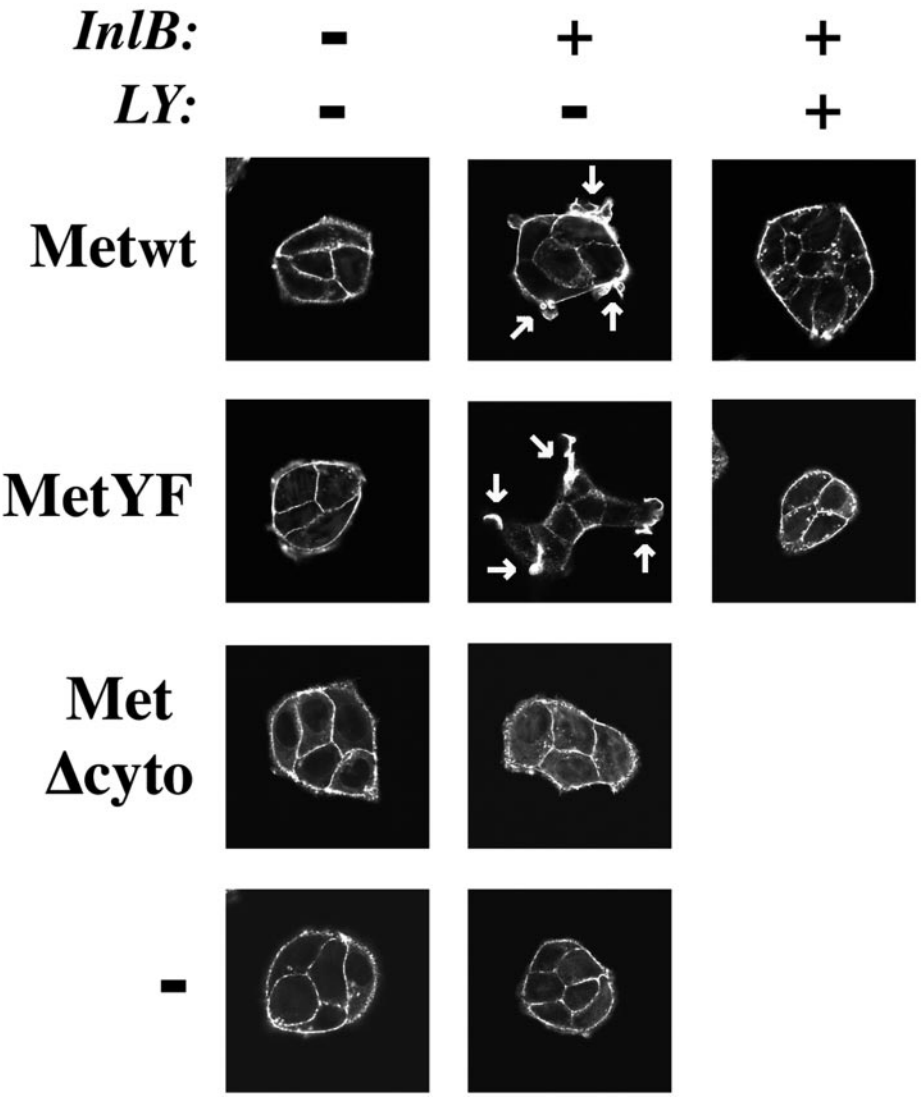

FIG. 2. InlB-induced membrane ruffling requires the cytoplasmic domain of Met but not tyrosines 1349 and 1356. T47D cell lines stably expressing Metwt, MetYF, or MetΔcyto were starved for ~5 hr in DMEM without FBS before preincubation in 100 μM LY294002 (+) or the vehicle Me_2SO (−) for 1 h. Cells were then stimulated or not with 1.5 nM InlB protein for 5 min before fixation and labeling of F-actin with Texas Red conjugated to phalloidin. Each cell line grows as islands of 4 to 10 cells, with cell borders being enriched for F-actin. Arrows indicate F-actin-rich membrane ruffles in cells treated with InlB. LY, LY294002.

ple buffer with 2.5% 2-mercaptoethanol and boiling for 5 min, and stored at −80°C.

Samples were migrated on SDS-polyacrylamide (7.5 or 9.0%) gels, transferred to polyvinylidene difluoride (PVDF) or nitrocellulose membranes, incubated with antibodies, and detected with an ECL, ECL Plus, or CDP Star chemiluminescence system (Amersham Biosciences) and exposure to film as described (17, 39). Unless otherwise stated, all immunoprecipitation or GST fusion protein precipitation experiments were performed at least three times, with similar results.

Laser scanning confocal microscopy. For analysis of membrane ruffling (Fig. 2), 1.5×10^5 T47D/Metwt, T47D/MetYF, T47D/MetΔcyto, or T47D cells were seeded on 22- by 22-mm glass coverslips in six-well plates and grown for ~2 days in DMEM with 10% FBS. For analysis of subcellular localization of EGFP-PH or HA-Gab1wt (see Fig. 6), cells were transfected as described above and grown for an additional ~24 h after addition of DNA-Lipofectamine 2000 complexes. Cells were then serum starved for ~5 h and incubated for 1 h in DMEM with 100 μM LY294002 or in DMEM with the vehicle Me_2SO (0.2%). Cells were stimulated by incubation with 100 ng of InlB protein per ml (1.5 nM) for 5 min at 37°C in 5% CO_2 or left unstimulated. Cells were then washed once in PBS and fixed in PBS with 3% paraformaldehyde. For membrane ruffling, fixed cells were permeabilized in PBS with 0.4% Triton X-100, followed by labeling of F-actin by a 1-h incubation in PBS with 0.5% BSA containing Texas Red X coupled to phalloidin. Cells transfected with pcDNA.HA-Gab1wt were permeabilized, followed by 1-h incubations in PBS with 0.5% BSA containing anti-HA antibody and then anti-mouse BODIPY FL. Cells transfected with EGFP or EGFP-PH did not require labeling. All coverslips were mounted on glass slides by using Mowiol 4-88 (catalog no. 475904; Calbiochem). Samples were analyzed by using a Zeiss LSM 510 microscope system equipped with a Zeiss Axioplan 2 microscope and argon (488 nm) and helium-neon (543 nm) lasers for detection of EGFP-BODIPY FL and Texas Red, respectively.

RESULTS

InlB-dependent entry of *Listeria* requires the cytoplasmic domain of Met but not tyrosines 1349 and 1356. In order to determine if the docking site tyrosines 1349 and 1356 in Met

are needed for InlB-mediated entry of *L. monocytogenes*, we constructed cell lines stably expressing either a truncated Met receptor deleted for most of its cytoplasmic domain (MetΔcyto) or a mutant receptor containing tyrosine-to-phenylalanine substitutions in both amino acids 1349 and 1356 (MetYF). The human breast cancer cell line T47D, which expresses very low levels of endogenous Met (33, 39) was used to generate the cell lines. Parental T47D cells are not susceptible to InlB-mediated entry, and stable expression of wild-type Met in these cells is sufficient to allow bacterial internalization (39). Therefore, T47D cells appeared to be suitable for complementation studies with MetΔcyto and MetYF.

Clones expressing the mutant Met receptors were analyzed for total receptor expression by quantitative Western blotting (Materials and Methods). Five clones each of T47D/MetΔcyto and T47D/MetYF cells that express receptor levels similar to those in our clone with wild-type Met (T47D/Metwt) were identified. Met surface expression in one of the T47D/MetΔcyto clones (clone 2) and one of the T47D/MetYF clones (clone 23) was assessed by flow cytometry and found to be at least as high as in T47D/Metwt cells (Materials and Methods).

T47D/MetΔcyto and T47D/MetYF cell lines were evaluated for the ability to support *Listeria* entry (Fig. 1) and mammalian signal transduction (see Fig. 2 to 9). Results for T47D/MetΔcyto-2 or T47D/MetYF-23 clones are presented, and similar results were obtained with the other independent clones (data not shown). Since T47D cells express E-cadherin, the receptor for the bacterial surface protein InlA, *L. monocytogenes* strains deleted of *inlA* (Δ*inlA*) or both *inlA* and *inlB* (Δ*inlAB*) were used to assess InlB-dependent entry (39). The Δ*inlA* strain entered into T47D/MetΔcyto cells at an efficiency that was similar to that in parental T47D cells and about sevenfold lower than that in T47D cells expressing wild-type Met (Fig. 1). In contrast to entry efficiency in T47D/MetΔcyto cells, entry of the Δ*inlA* strain in T47D/MetYF cells was similar in efficiency to that in T47D/Metwt cells. Given the demonstrated importance of tyrosines 1349 and 1356 in HGF-induced scattering and morphogenesis, we were somewhat surprised that entry into T47D/MetYF cells was nearly as efficient as that into cells expressing wild-type Met. Therefore, the presence of the tyrosine-to-phenylalanine mutations in the T47D/MetYF-23 cell line was confirmed by recovery of the 3′ end of the transfected Met cDNA from the chromosome by PCR and DNA sequencing (Materials and Methods). Taken together, the results shown in Fig. 1 indicate that the cytoplasmic domain of Met is required for bacterial internalization, but phosphorylation of tyrosines 1349 and 1356 is not absolutely essential.

Docking site tyrosines in Met are not needed for InlB-induced membrane ruffling. Incubation of mammalian cells with soluble InlB protein induces changes in the F-actin cytoskeleton and cell motility. In the African green monkey kidney cell line Vero, InlB treatment results in rapid membrane ruffling and lamellipodia formation (17). In MDCK cells, prolonged stimulation with InlB induces cell scattering in a manner similar to that caused by HGF (39). InlB-induced membrane ruffling and cell scattering are both dependent on PI 3-kinase activity, since these events are blocked by treatment with the PI 3-kinase inhibitors wortmannin or LY294002.

Similar to the results with Vero cells, incubation of T47D/Metwt cells with InlB (1.5 nM) caused formation of membrane ruffles enriched in F-actin, as detected by staining with fluorescently labeled phalloidin (Fig. 2). These ruffles formed rapidly (within 5 min) at the lateral or dorsal surfaces of cell islands. In contrast to T47D cells expressing wild-type Met, neither the parental T47D nor the T47D/MetΔcyto cell lines formed membrane ruffles upon incubation with InlB. However, InlB was able to elicit membrane ruffling in the T47D/MetYF cell line. The results shown in Fig. 2 indicate that InlB-induced cytoskeletal changes in T47D cells require the cytoplasmic domain of Met but not phosphorylation of tyrosines 1349 and 1356, which is similar to the requirements for *Listeria* entry (Fig. 1).

Tyrosines 1349 and 1356 in Met are needed for recruitment of Grb2, p85, and the Gab1 MBD, but not for Gab1 phosphorylation. The MetΔcyto receptor lacks the kinase domain and all known phosphorylation sites, except tyrosine 1003. As expected, tyrosine phosphorylation of this mutant receptor could not be detected in T47D/MetΔcyto cells upon stimulation (Fig. 3). In contrast, the receptor in T47D/MetYF cells was tyrosine phosphorylated, indicating that the docking site tyrosines are not needed for Met activation. Phosphorylation of the MetYF receptor likely occurs at the activation loop tyrosines 1234 and 1235 and possibly tyrosine Y1003. Consistent with these results in T47D cells, activation (phosphorylation) of Met mutated at tyrosines 1349 and/or 1356 was previously observed in COS-1 and MDCK cells (11, 48).

When expressed in COS-1, 293T, MDCK, and other cell lines, tyrosine-to-phenylalanine mutations in amino acids 1349 and 1356 in Met abolish HGF-induced recruitment of several SH2 domain-containing effector proteins, including p85 and Grb2 (11, 26, 35, 42, 48). Recruitment of the MBD in Gab1 to Met is also inhibited by mutation of the docking site tyrosines in the receptor (1, 23, 24, 26, 44). In accordance with published data on other cell lines, in T47D cells tyrosines 1349 and 1356 were needed for efficient InlB-, HGF-, or bacterial-induced association of Met with the SH2 domains of Grb2 or p85 and with the MBD of Gab1 (Fig. 4 and data not shown). These results demonstrate that receptor-proximal signaling events involving recruitment of SH2 domain signaling proteins are defective in T47D/MetYF cells as well as in T47D/MetΔcyto cells.

In contrast to these receptor-proximal signaling events, more distal signaling events were not abolished in T47D/MetYF cells. InlB- or HGF-induced phosphorylation of ERK-1/2 were normal in cells expressing MetYF (Fig. 5A), indicating that the docking site tyrosines in Met were not required for activation of the MAPK pathway. Full HGF-induced activation of MAPK is known to require tyrosine phosphorylation of the adapter protein Gab1 (18, 29). Importantly, InlB-, HGF-, or bacterial-mediated phosphorylation of Gab1 in T47D cells was not fully eliminated by mutation of tyrosines 1349 and 1356 (Fig. 5B and see Fig. 8A). The extent of Gab1 phosphorylation in T47D/MetYF cells in response to these three stimuli was variable, at times appearing nearly as efficient as in cells expressing wild-type Met (see Fig. 8A) and at other times exhibiting a clear decrease relative to cells with wild-type receptor (Fig. 5B). The reason for this variability is not known. In summary, Gab1 phosphorylation was not abolished by mutation of the docking site tyrosines. In contrast to activation in cells expressing MetYF, activation of MAPK (Fig. 5A) and phosphorylation of

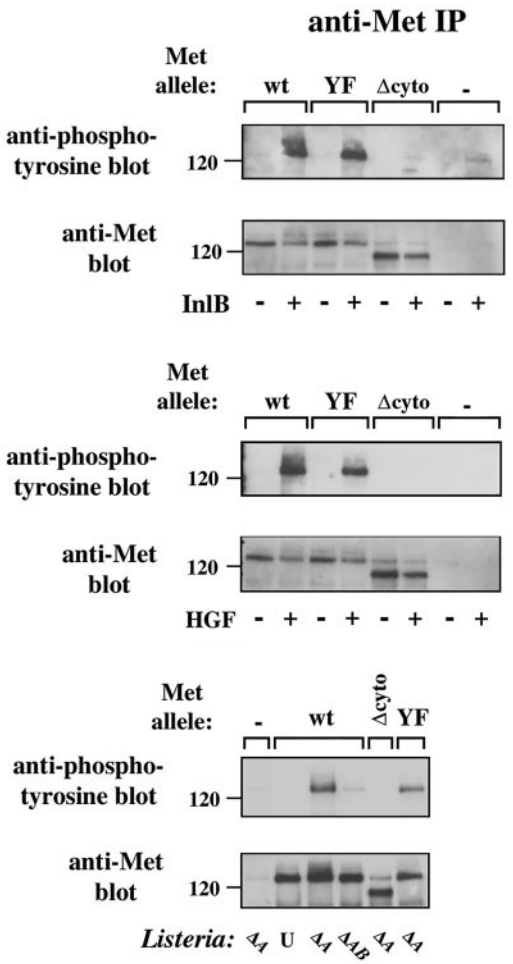

FIG. 3. Phosphorylation of wild-type and mutant Met receptors in T47D cell lines. Cells were serum starved and either treated with InlB or HGF or infected with *L. monocytogenes* strains as described in Materials and Methods. After solubilization, cell lysates were subjected to immunoprecipitation (IP) with an antibody recognizing the extracellular domain of Met (DO-24), and immunoprecipitates were migrated on SDS-PAGE gels, transferred to PVDF membranes, and subjected to Western blotting with antibodies specific for phosphotyrosine (upper panel). Membranes were then stripped and probed with anti-Met antibody DL-21, which recognizes an epitope in the extracellular domain of the receptor. U, uninfected cells; Δ*A*, cells infected with the Δ*inlA* mutant of *Listeria*; Δ*AB*, cells infected with the Δ*inlAB* bacterial mutant. We note that the low-level phosphorylation of wild-type Met in cells infected with the Δ*inlAB* bacterial strain was not routinely observed in two other independent experiments.

Gab1 were defective in cells expressing MetΔcyto (Fig. 5B) and in parental T47D cells (Fig. 5B and data not shown).

The PH domain of Gab1 is constitutively associated with the plasma membrane of T47D cells. In one previous study examining tyrosines 1349 and 1356 in Met, mutation of these residues to phenylalanine was found to cause a nearly complete defect in Met-dependent Gab1 phosphorylation (42). However, in other reports significant Gab1 phosphorylation was detected in cells expressing Met in which the docking site tyrosines were mutated or deleted (13, 26). The reasons for these differences in Gab1 phosphorylation are not understood but may be due to the different cell lines employed. We sought to understand the mechanism by which Gab1 is phosphorylated in the absence of the docking site tyrosines in Met.

HGF-induced phosphorylation of Gab1 is believed to require recruitment of the adapter protein to the plasma membrane. One pathway of membrane recruitment involves association with the phosphorylated docking site tyrosines in Met (1, 23, 24, 31). Association with these tyrosines occurs both indirectly through binding of Grb2 in Grb2-Gab1 complexes and directly via interaction with the Gab1 MBD. A second pathway of membrane recruitment is promoted by interaction of the Gab1 PH domain with PIP3 in the inner leaflet of the plasma membrane (27, 28). Since our results indicated that Grb2- and MBD-mediated association of Gab1 with Met was defective in T47D/MetYF cells (Fig. 4), we considered the possibility that Gab1 phosphorylation in these cells might be mediated by PH domain-dependent membrane recruitment.

In order to detect membrane recruitment of the Gab1 PH domain, a gene encoding a chimeric protein consisting of the PH domain fused to the carboxyl-terminus of EGFP was constructed and transiently expressed in T47D/Metwt and T47D/MetYF cells. Fluorescence microscopy analysis indicated that EGFP-PH was localized predominantly at cell borders in serum-starved cells that were not treated with InlB (Fig. 6A). These results suggest that the PH domain is constitutively associated with the plasma membrane in these cells. Similar constitutive membrane association of EGFP-PH was also observed in T47D/MetΔcyto and T47D cells (data not shown). Addition of soluble InlB protein (1.5 nm for 5 min) resulted in translocation of EGFP-PH to membrane ruffles. Both the constitutive localization of EGFP-PH to cell borders and the InlB-induced redistribution to ruffles were abolished by incubation of cells with the PI 3-kinase inhibitor LY294002. These latter results suggest that association of the Gab1 PH domain with the plasma membrane required interaction with PIP3. In order to determine the contribution of sequences outside of the PH domain to membrane recruitment, we also analyzed localization of full-length HA-tagged Gab1 in T47D/Metwt and T47D/MetYF cells (Fig. 6A). Similar to the isolated PH domain, full-length Gab1 was constitutively associated with the plasma membrane in a PI 3-kinase-dependent fashion in both cell lines. InlB-induced recruitment of Gab1 to membrane ruffles was blocked by LY294002, as expected from the absence of ruffles in cells treated with this inhibitor (Fig. 2). However, membrane recruitment of full-length Gab1 in response to InlB was not blocked by inhibition of PI 3-kinase activity in T47D cells expressing wild-type Met. HA-tagged Gab1 localized to cell borders in T47D/Metwt cell lines treated with both LY294002 and InlB. In contrast to results in cells expressing wild-type Met, in cells expressing MetYF, LY294002 fully inhibited the InlB-induced membrane association of Gab1. Taken together, the experiments with EGFP-PH and full-length Gab1 suggest two potential pathways of membrane recruitment for Gab1 in T47D cells. One pathway is mediated by interaction of the Gab1 PH domain with membrane PIP3. This mode of recruitment is dependent on PI 3-kinase activity and is constitutive in both T47D/Metwt and T47D/MetYF cells. A second recruitment pathway that is induced by InlB and inde-

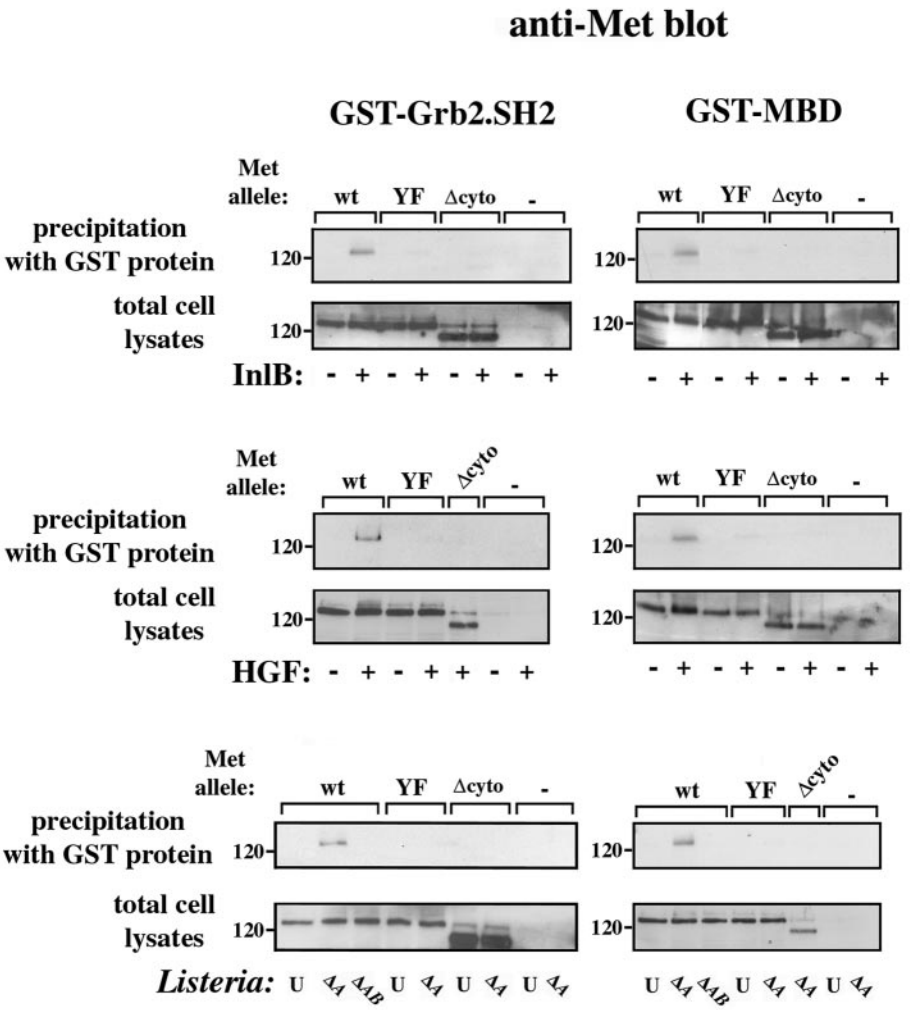

FIG. 4. Tyrosines 1349 and 1356 are needed for efficient recruitment of the Grb2 SH2 domain or the Gab1 MBD to Met. After treatment with InlB or HGF or infection with *L. monocytogenes* strains, cells were solubilized in lysis buffer and lysates were used for precipitation with GST fusion proteins containing either the SH2 domain of Grb2 or the Gab1 MBD. Precipitates were subjected to Western blotting with antibody DL-21. Control precipitations with GST alone did not result in coprecipitation of Met from lysates of cells stimulated with InlB, HGF, or bacteria (data not shown). U, uninfected cells; Δ*A*, cells infected with the Δ*inlA* mutant of *Listeria*; Δ*AB*, cells infected with the Δ*inlAB* bacterial mutant.

pendent of PI 3-kinase activity is operative in cells expressing wild-type Met but not in cells expressing MetYF. This pathway probably involves direct or indirect association of Gab1 with Met.

The fact that membrane localization of EGFP-PH was ligand independent but PI 3-kinase dependent suggested that PI 3-kinase activity was constitutive in the T47D cell lines. Consistent with this idea, robust phosphorylation of serine 473 in the PI 3-kinase target Akt was observed in serum-starved T47D/Metwt, T47D/MetYF, T47D/MetΔcyto, and T47D cells that were not subjected to stimulation with InlB (Fig. 7A and data not shown). Treatment of these cell lines with LY294002 eliminated Akt phosphorylation, indicating PI 3-kinase dependency. For comparison, we analyzed Gab1 PH domain localization and Akt phosphorylation in Vero cells, a monkey kidney cell line that expresses Met and is known to have basal PIP3 levels that are low to undetectable (16, 17). The EGFP-PH fusion was not detected at the plasma membrane in the absence of stimulation in Vero cells but, instead, was predominantly localized to the nucleus (Fig. 6B). Similar nuclear distribution of a Gab1 PH domain EGFP fusion protein was previously observed in unstimulated COS-1 cells (37), although the reason for such localization is not known. Upon treatment of Vero cells with InlB, the isolated Gab1 PH domain translocated to membrane ruffles (Fig. 6B). Similarly, Akt phosphorylation was low to undetectable in untreated cells and became prominent upon addition of InlB (Fig. 7B). InlB-induced redistribution of the Gab1 PH domain and Akt phosphorylation were both inhibited by treatment of cells with LY294002, indicating a requirement for PI 3-kinase in these events. Taken together, the results shown in Fig. 6 and 7 demonstrate that the PH domain of Gab1 is constitutively associated with the plasma membrane of T47D/Metwt and T47D/MetYF cells in a manner that is dependent on PI 3-kinase activity.

In order to determine if the constitutive plasma membrane

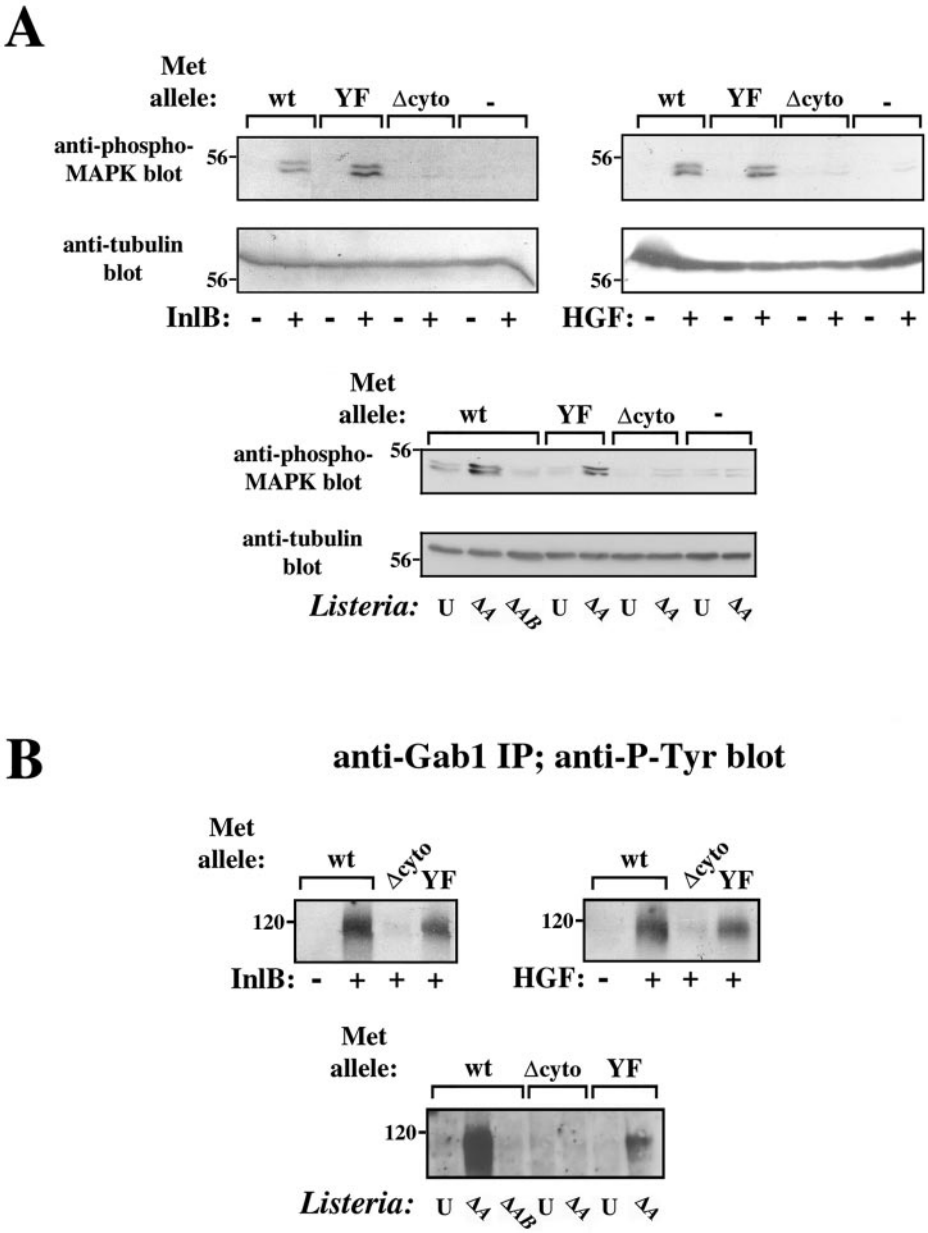

FIG. 5. Tyrosines Y1349 and 1356 are not needed for InlB-induced MAPK or Gab1 phosphorylation. (A) MAPK phosphorylation in response to InlB, HGF, or *Listeria* in cells expressing Metwt, MetYF, or MetΔcyto. Total cell lysates were assessed for MAPK activation by immunoblotting with a phospho-specific antibody that recognizes phosphorylated ERK-1/2 isoforms (upper panel). Membranes were stripped and probed with an anti-tubulin antibody to assess gel loading (lower panel). (B) Gab1 phosphorylation in response to InlB, HGF, or *Listeria*. After stimulation with InlB, HGF, or bacteria, cells were solubilized in lysis buffer, and immunoprecipitation (IP) was performed with anti-Gab1 antibodies. Immunoprecipitates were subjected to Western blotting with anti-phosphotyrosine antibodies. U, uninfected cells; Δ*A*, cells infected with the Δ*inlA* mutant of *Listeria*; Δ*AB*, cells infected with the Δ*inlAB* bacterial mutant.

recruitment of Gab1 observed in T47D cells was needed for bacterial-induced phosphorylation, we used LY294002 to inhibit PI 3-kinase activity. Incubation with LY294002 impaired InlB-, HGF-, or bacterial-induced Gab1 phosphorylation in T47D/MetYF cells but not in T47D/Metwt cells (Fig. 8A) or in Vero cells (data not shown). This inhibitory effect of LY294002 on Gab1 phosphorylation was observed in each of the four other clones of T47D/MetYF cells that we tested (data not shown), indicating that it is not an aberrant phenotype confined to a particular clone. Importantly, InlB- or bacterial-induced activation of MAPK was also inhibited by treatment of T47D/MetYF cells with LY294002 (Fig. 8B). In contrast, inhibition of PI 3-kinase with this chemical did not eliminate MAPK activation in T47D/Metwt cells. Collectively, the results shown in Fig. 6 to 8 support the idea that membrane recruitment of Gab1 through its PH domain is critical for bacterial-induced Gab1 phosphorylation and MAPK activation in T47D cells expressing MetYF.

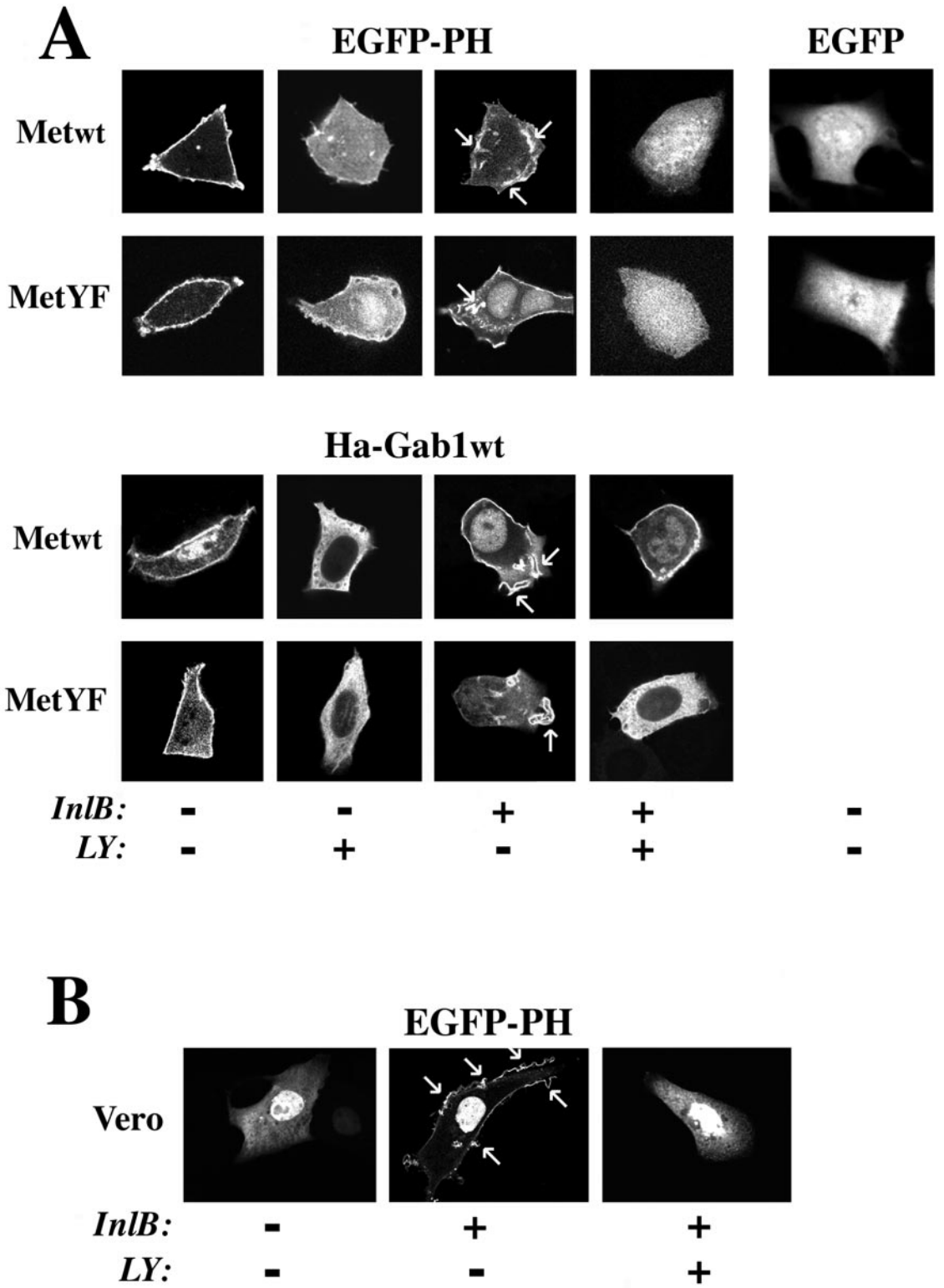

FIG. 6. The Gab1 PH domain is constitutively associated with the plasma membrane in T47D cells. (A) Localization of an EGFP-Gab1 PH domain fusion (EGFP-PH), control EGFP, or full-length HA-tagged Gab1 (HA-Gab1wt) in T47D cells. T47D/Metwt or T47D/MetYF cell lines were transiently transfected with plasmids encoding a fusion between EGFP and the Gab1 PH domain (EGFP-PH), EGFP alone, or HA-Gab1wt. Cells were then serum starved for ~5 h before incubation in DMEM containing the PI 3-kinase inhibitor LY294002 (+) or the vehicle Me_2SO (−) for 1 h, followed by stimulation with 1.5 nM InlB for 5 min. (B) Localization of the EGFP-Gab1 PH domain fusion in Vero cells. Vero cells were transiently transfected with a plasmid expressing EGFP-PH, serum starved for ~4 h, incubated in DMEM with LY294002 (+) or Me_2SO (−) for ~45 min, and then treated (+) or not (−) with 1.5 nM InlB protein for 5 min. Arrows in panels A and B indicate EGFP-PH or HA-Gab1wt localized to membrane ruffles. LY, LY294002.

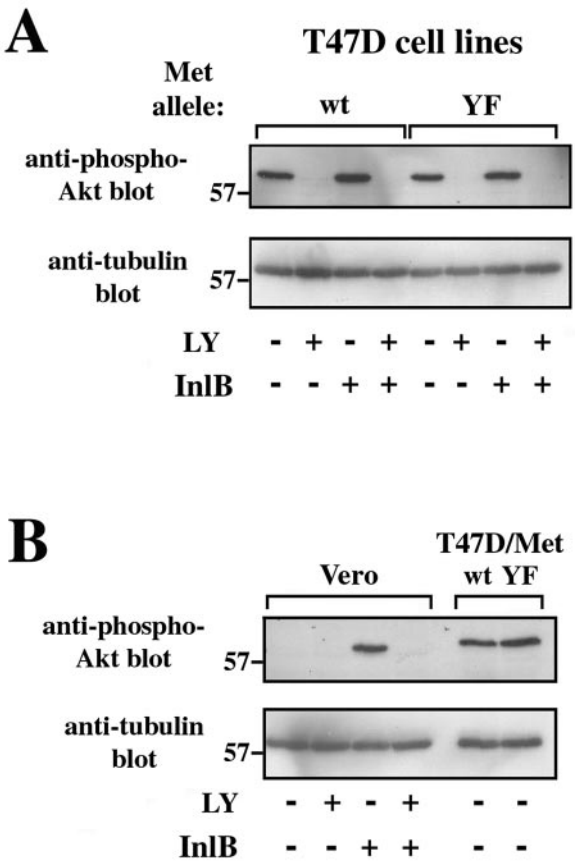

FIG. 7. The PI 3-kinase target Akt is constitutively phosphorylated in T47D cells. (A) Phosphorylation of Akt in T47D cell lines. T47D/Metwt or T47D/MetYF cells were serum starved for ~16 h and incubated in DMEM with LY294002 (+) or vehicle (−), and total cell lysates were analyzed for phosphorylation of serine 473 in Akt (upper panel). To verify loading, membranes were stripped and probed with anti-tubulin antibody (lower panel). (B) A comparison of Akt phosphorylation in T47D and Vero cells. Equal protein amounts from total cell lysates of serum-starved Vero or T47D cell lines were analyzed for Akt phosphorylation. LY, LY294002.

PH domain-mediated recruitment of Gab1 is needed for bacterial entry in the absence of tyrosines 1349 and 1356. In order to determine if PH domain-dependent recruitment of Gab1 is required for *Listeria* entry, the effect of overexpression of EGFP-PH on bacterial internalization into T47D/Metwt and T47D/MetYF cells was tested. We reasoned that overexpression of the isolated PH domain might displace endogenous Gab1 from the membrane and serve as a tool for examining the role of PH domain-mediated recruitment. Overexpression of EGFP-PH blocked entry of the Δ*inlA* strain of *L. monocytogenes* into T47D/MetYF cells, while leaving entry into T47D/Metwt cells virtually unaffected (Fig. 9, left panel). Cotransfection of EGFP-PH with HA-tagged wild-type Gab1 suppressed the defect in bacterial entry normally caused by transfection with EGFP-PH alone. The latter result indicates that the defect in internalization caused by overexpression of EGFP-PH was due to effects on Gab1 and not another PH domain-containing protein. As a control for this suppression experiment, cells were cotransfected with EGFP-PH and a tagged mutant Gab1 allele deleted of the PH domain (Ha-Gab1ΔPH). In contrast to the situation with wild-type Gab1, the Gab1ΔPH allele failed to localize to the plasma membrane of T47D cell lines (data not shown). As expected, overexpression of Gab1ΔPH did not restore bacterial entry to T47D/MetYF cells coexpressing the isolated PH domain. The inhibitory effect of EGFP-PH on internalization in cells expressing MetYF was not due to alterations in binding of *Listeria* to host cells (Fig. 9, right panel). Taken together, the findings indicate that PH domain-mediated recruitment of Gab1 is essential for entry of *Listeria* into T47D cells expressing Met mutated for the docking site tyrosines. When tyrosines 1349 and 1356 are present, PH domain-dependent recruitment of Gab1 becomes dispensable for entry. The two pathways of Gab1 membrane association are functionally redundant (Fig. 10).

DISCUSSION

When phosphorylated, tyrosines 1349 and 1356 in the cytoplasmic tail of Met serve as multisubstrate docking sites for Grb2, p85, PLC-γ, and other SH2 domain-containing proteins (11, 35, 48). HGF-induced recruitment of the MBD in Gab1 also requires phosphorylation of these docking site tyrosines (1, 24, 26). Mutation of the tandem tyrosines to phenylalanine results in defects in ligand-induced scattering and morphogenesis of cultured cells (11, 48) and in developmental defects in mice that closely resemble those caused by a *met* null mutation (25). These results indicate that phosphorylation of tyrosines 1349 and 1356 plays a critical role in HGF-induced biological events.

In this work, InlB-mediated internalization of *Listeria* into the human breast cancer epithelial cell line T47D was found to require the cytoplasmic domain of Met. Surprisingly, the docking site tyrosines were not absolutely essential for *Listeria* entry or for bacterial-induced Gab1 phosphorylation. Gab1 was phosphorylated in cells expressing MetYF, despite a defect in recruitment of both Grb2 and the Gab1 MBD to this receptor. Instead, phosphorylation of Gab1 in these cells required host PI 3-kinase activity, reflecting a requirement for PIP3 in constitutive membrane association of the Gab1 PH domain. Experiments in which the Gab1 PH domain was overexpressed indicated that PH domain-mediated membrane recruitment was required for entry in cells expressing MetYF. In contrast, PH domain-dependent recruitment was not essential for bacterial internalization in cells expressing Metwt. Taken together, these results indicate redundant functions for receptor- and PH domain-mediated recruitment of Gab1 in uptake of *Listeria* (Fig. 10).

It is worth noting that experiments with the inhibitor LY294002 indicate that host PI 3-kinase activity is required for bacterial entry into both T47D/Metwt and T47D/MetYF cells (Fig. 9), despite the fact that Gab1 phosphorylation is normal in T47D/Metwt cells. These results indicate that PI 3-kinase activity regulates *Listeria* entry by acting both upstream and downstream of Gab1. The precise downstream function of PI 3-kinase is unclear. However, by analogy to the known role of this lipid kinase in classic phagocytosis mediated by the Fc-γ receptor (8), it is possible that one of the functions of PI 3-kinase in InlB-mediated entry might be to promote extension

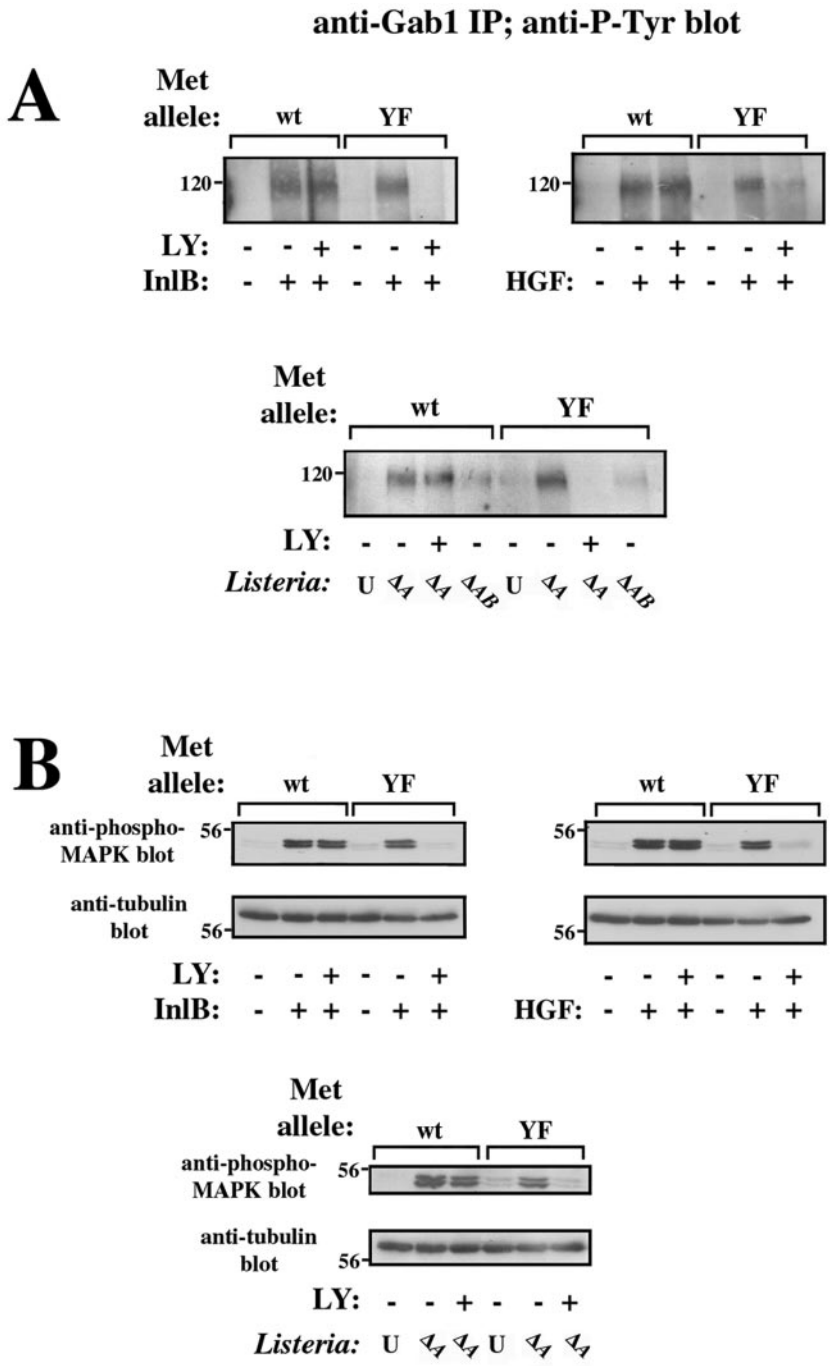

FIG. 8. InlB-induced phosphorylation of Gab1 in the absence of tyrosines 1349 and 1356 in Met is PI 3-kinase-dependent. (A) The effect of LY294002 treatment on Gab1 phosphorylation in cell lines expressing Metwt or MetYF. Cells were preincubated with LY294002 before treatment with InlB, HGF, or infection with *Listeria* strains. Gab1 was immunoprecipitated from lysates, and its phosphorylation state was evaluated by Western blotting with anti-phosphotyrosine antibodies. (B) The effect of LY294002 on MAPK phosphorylation. Total cell lysates from cells incubated with InlB, HGF, or *Listeria* were migrated on SDS-PAGE gels, transferred to PVDF, and reacted with anti-phospho-MAPK antibodies (upper panel). After stripping, membranes were probed with anti-tubulin antibodies (lower panel). We note that a small amount of Gab1 phosphorylation occurred in T47D/Metwt or T47D/MetYF cell lines infected with the Δ*inlAB* bacterial strain (panel A). This residual effect on Gab1 implies the existence of bacterial factors in addition to InlB that are capable of stimulating host signal transduction. The identity of such factor(s) is not known. Similar low-level, InlB-independent phosphorylation of Gab1 was previously observed in Vero cells (17). U, uninfected cells; Δ*A*, cells infected with the Δ*inlA* mutant of *Listeria*; Δ*AB*, cells infected with the Δ*inlAB* bacterial mutant; LY, LY294002.

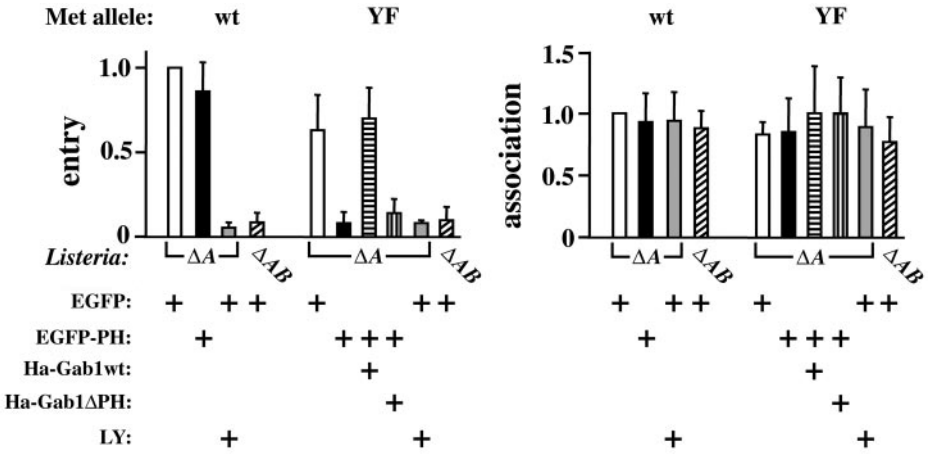

FIG. 9. Entry of *Listeria* in cell lines expressing MetYF is blocked by overexpression of the Gab1 PH domain. Cell lines expressing Metwt or MetYF were transiently transfected with a plasmid expressing EGFP fused to the PH domain of Gab1 (EGFP-PH) or a plasmid expressing EGFP alone or cotransfected with plasmids overexpressing EGFP-PH and HA-tagged full-length Gab1 (HA-Gab1wt) or tagged Gab1 deleted of its PH domain (HA-Gab1ΔPH). In some experiments, cells transiently expressing EGFP-PH were incubated with LY294002 to inhibit host cell PI 3-kinase activity. Cells were then infected with the indicated Δ*inlA* (Δ*A*) or Δ*inlAB* (Δ*AB*) mutant strain of *Listeria* as described in Materials and Methods. Entry (left panel) indicates the relative number of intracellular bacteria per host cell, and association (right panel) indicates the relative number of total (intracellular and extracellular) bacteria associated per host cell. The data are average values ± standard deviations from two to four experiments, depending on the condition. In each experiment, 50 to 140 transfected host cells were analyzed. Data from each experiment were first expressed as the average number of intracellular bacteria or intracellular plus extracellular bacteria per mammalian cell. In four experiments, the absolute entry values for the Δ*inlA* bacterial strain in T47D/Metwt cells transfected with EGFP ranged from 0.4 to 1.2 bacteria/cell. The corresponding absolute association values were between 1.2 and 3.1 bacteria/cell. Absolute values from each experiment were converted to relative entry and association by normalizing to entry or association of the Δ*inlA* strain in T47D/Metwt cells transfected with EGFP (white bars). LY, LY294002.

and sealing of pseudopodia (finger-like projections of the host plasma membrane) around adherent *Listeria*.

Overexpression of the Gab1 PH domain, which is known to bind PIP3, inhibited *Listeria* entry specifically into cell lines expressing MetYF. Type IA PI 3-kinase generates both PIP3 and phosphatidylinositol 3,4-bisphosphate [$PI(3,4)P_2$], and it is not presently known whether it is PIP3, $PI(3,4)P_2$, or both lipids that promote internalization of *Listeria*. The Gab1 PH domain binds with highest affinity to PIP3, and with lower affinity to $PI(3,4)P_2$ (27, 37). It seems likely that the inability of the overexpressed PH domain to block bacterial internalization into cells expressing wild-type Met is due to incomplete saturation of membrane PIP3 and/or $PI(3,4)P_2$.

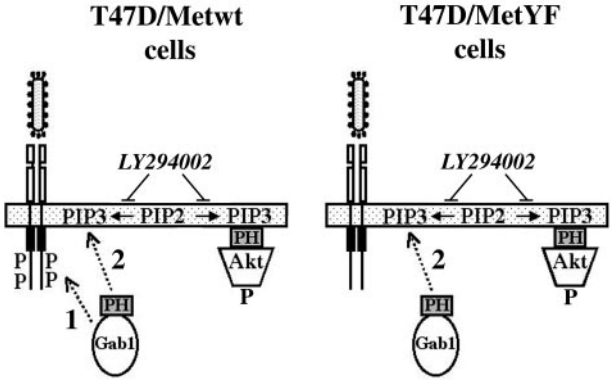

FIG. 10. Phosphorylated tyrosines in Met and the Gab1 PH domain play redundant roles in InlB-dependent entry into T47D cells. Gab1 phosphorylation and entry in cell lines expressing Metwt or MetYF can be explained according to the following model. Two pathways of membrane association exist in cell lines expressing Metwt: (i) recruitment of Gab1 to phosphorylated tyrosines 1349 and 1356 and (ii) PH domain-dependent recruitment of Gab1 to PIP3 in the membrane. Immunofluorescence microscopy data (Fig. 6) indicate that, in cell lines expressing MetYF, only the second pathway is operative. Conditions expected to inhibit docking of the Gab1 PH domain to PIP3 (treatment with LY294002 or overexpression of the isolated PH domain) inhibit bacterial-induced Gab1 phosphorylation or entry.

PH domain-mediated membrane association of Gab1 has previously been observed in MDCK, COS-1, and Vero cells (27, 28, 37, 45). In these cells, membrane localization is ligand dependent, since it is weak or undetectable in the absence of serum and increases dramatically upon incubation with serum, HGF, or EGF. In contrast, PH domain-mediated recruitment of Gab1 appeared to be constitutive (ligand independent) in T47D cells, as it was detected in serum-starved cells that were not treated with InlB. Ligand-independent association of Gab1 with the plasma membrane required PI 3-kinase activity. Analysis of Akt phosphorylation suggested that PI 3 kinase activity is also likely to be constitutive in serum-starved T47D cell lines. Prolonged starvation in the absence of serum for up to 48 h failed to abolish association of the Gab1 PH domain with the plasma membrane or to reduce phosphorylation of serine 473 in Akt (T. Basar, Y. Shen, and K. Ireton, unpublished results). In addition, growth of cells for several generations in dialyzed serum depleted of EGF and other small (<10 kDa) molecules did not substantially reduce Gab1 membrane association or Akt phosphorylation. Based on these results, the constitutive PI 3-kinase activation in T47D cells is probably not due to serum-dependent activation of signaling pathways. In agreement with our findings, Nicholson and coworkers recently reported constitutive phosphorylation of Akt in T47D cells that

was dependent on PI 3-kinase activity and independent of serum (32). Their data suggest that Akt phosphorylation may be due to production of autocrine factors that activate the EGF receptor family members ErbB1 and/or ErbB2. Taken together with our results, these findings raise the interesting possibility that downstream signaling through Met may be influenced by the activation state of other receptor tyrosine kinases.

The requirement for the Met docking site tyrosines in Gab1 phosphorylation appears to be cell line dependent. Our results indicate that tyrosines 1349 and 1356 are not essential in T47D cells because of constitutive membrane association of Gab1. Similarly, these two tyrosines are not absolutely required for Gab1 phosphorylation in mouse hepatocytes expressing physiological levels of Met or in COS-7 cells transiently expressing the Tpr-Met oncoprotein. Mutation of tyrosines 1349 and 1356 reduces, but does not abolish, Gab1 phosphorylation in these two cell lines (13, 26). In contrast to the situation in T47D, COS-7 cells, or hepatocytes, in MDCK cells the two tyrosines are essential for Gab1 phosphorylation (42). It would be interesting to determine if the residual phosphorylation of Gab1 observed in mouse hepatocytes or COS-7 cells is due to PH domain-dependent recruitment of Gab1.

Consistent with the findings in the present study, results from previous studies also point to a role for the PH domain in Gab1 phosphorylation or function downstream of Met or other surface receptors. In the case of the B-cell antigen receptor (BCR), the PH domain in Gab1 is essential for phosphorylation and function of this adapter protein (15). The absolute requirement for this domain is probably due to the fact that the BCR and associated membrane proteins lack phosphorylation sites able to directly recruit Gab1. In contrast to the BCR, Met or the EGF receptor directly associates with Gab1, allowing dual pathways of membrane recruitment. In MDCK cells, overexpression of wild-type Gab1 suppresses the defect in branching tubulogenesis normally caused by a mutation in Met that prevents recruitment of Grb2 and Gab1 (28). Gab1 deleted of its PH domain fails to suppress this tubulogenesis defect, indicating that PH domain-mediated membrane recruitment is critical in the absence of association with Met. The PH domain is also needed for full EGF-induced phosphorylation of Gab1 (45) and Jun N-terminal protein kinases activation (37). These findings for Met and the EGF receptor are conceptually similar to our results, suggesting that Gab1 can be regulated through redundant pathways involving recruitment to Met or to PIP3 in the plasma membrane.

Finally, our results with overexpression of the Gab1 PH domain suggest that Gab1 likely participates in InlB-dependent entry of *Listeria*. A role for Gab1 in bacterial uptake is confirmed by a recent study from our laboratory, in which Gab1 was found to promote entry through association with p85 and Crk family proteins (40).

ACKNOWLEDGMENTS

We are grateful to M. Park (McGill University) for generous gifts of wild-type and mutant Met cDNAs, as well as GST fusion protein constructs. We thank M. Holgado-Madruga and A. Wong (Kimmel Cancer Institute) for HA-tagged Gab1 constructs and S. Gray-Owen and A. Wilde for providing comments on the manuscript.

This work was supported by grants from the Canadian Institutes of Health Research (MT-15497) and the Ontario Ministry of Energy, Science, and Technology (Premier's Research Excellence Award) awarded to K.I. K.I. is a recipient of a Canadian Institutes of Health Research (CIHR) New Investigator Award.

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Editor: V. J. DiRita