Quantitative Phosphokinome Analysis of the Met Pathway Activated by the Invasin Internalin B from Listeria monocytogenes*

Tobias Reinl‡, Manfred Nimtz‡, Claudia Hundertmark‡, Thorsten Johl‡, György Kéri§¶, Jürgen Wehland‡, Henrik Daub¶, and Lothar Jänsch‡**

Stimulated by its physiological ligand, hepatocyte growth factor, the transmembrane receptor tyrosine kinase Met activates a signaling machinery that leads to mitogenic, motogenic, and morphogenic responses. Remarkably, the food-borne human pathogen Listeria monocytogenes also promotes autophosphorylation of Met through its virulence factor internalin B (InlB) and subsequently exploits Met signaling to induce phagocytosis into a broad range of host cells. Although the interaction between InlB and Met has been studied in detail, the signaling specificity of components involved in InlB-triggered cellular responses remains poorly characterized. The analysis of regulated phosphorylation events on protein kinases is therefore of particular relevance, although this could not as yet be characterized systematically by proteomics. Here, we implemented a new pyridopyrimidine-based strategy that enabled the efficient capture of a considerable subset of the human kinome in a robust one-step affinity chromatographic procedure. Additionally, and to gain functional insights into the InlB/Met-induced bacterial invasion process, a quantitative survey of the phosphorylation pattern of these protein kinases was accomplished. In total, the experimental design of this study comprises affinity chromatographic procedures for the systematic enrichment of kinases, as well as phosphopeptides; the quantification of all peptides based on the iTRAQ™ reporter system; and a rational statistical strategy to evaluate the quality of phosphosite regulations. With this improved chemical proteomics strategy, we determined and relatively quantified 143 phosphorylation sites detected on 94 human protein kinases. Interestingly, InlB-mediated signaling shows striking similarities compared with the natural ligand hepatocyte growth factor that was intensively studied in the past. In addition, our systematic approach suggests a new subset of protein kinases including Nek9, which are differentially phosphorylated after short time (4-min) treatment of cells with the Met-activating InlB

The human food-borne pathogen Listeria monocytogenes has evolved mechanisms to cross the intestinal, placental, and blood-brain barriers with severe consequences for pregnant women, newborns, and immunocompromised individuals. As a facultative intracellular pathogen, L. monocytogenes invades host cells within minutes, thus escaping the humoral arm of adaptive immunity. In this protective host niche, the organism replicates and spreads from cell to cell through the formation of so-called membrane protrusions. L. monocytogenes utilizes two different molecular routes to invade non-professional phagocytic cells. (i) Internalin A binds to the cell adhesion molecule E-cadherin, resulting in the initial penetration of intestinal tissue (1, 2). (ii) In contrast, internalin B (InlB) contributes to the systemic infection of the host, promoting the invasion of a broader range of cell types including hepatocytes (3) and endothelial cells (4). A basic GW motif at the C terminus mediates the attachment of InlB to the bacterial cell wall, but the non-covalent nature of this interaction also al-

1 The abbreviations used are: InlB, internalin B; HG, hepatocyte growth factor; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; Erk, extracellular signal-regulated kinase (Erk1 = MK03, Erk2 = MK01); GSK3, glycogen synthase kinase 3; Mek, dual specificity mitogen-activated protein kinase/extracellular signal-regulated kinase (Mek1 = MP2K1, Mek2 = MP2K2); PTM, posttranslational modification; iTRAQ, isobaric tag for relative and absolute quantification; SILAC, stable isotope labeling by amino acids in cell culture; RF, regulation factor; SMAC, small molecule affinity chromatography; GK, gatekeeper; RP, reversed phase; MAPK, mitogen-activated protein kinase; DMF, N,N-dimethylformamide; EDC-HCl, N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride; DMEM, Dulbecco’s modified Eagle’s medium; HRP, horseradish peroxidase; SPE, solid phase extraction; SCX, strong cation exchange; UPLC, ultraperformance LC; FP, false positive; TP, true positive; p, phospho-; MARK, microtubule-associated protein/microtubule affinity-regulating kinase; AMPK, AMP-activated protein kinase; MT, microtubule.

From the ‡Department of Cell Biology, Helmholtz Centre for Infection Research, 38124 Braunschweig, Germany, §Vichem Chemie Ltd., Budapest 1022, Hungary, ¶Peptide Biochemistry Research Group, Department of Medicinal Chemistry, Semmelweis University, Budapest 1088, Hungary, and ⅡCell Signaling Group, Department of Molecular Biology, Max Planck Institute of Biochemistry, 82152 Martinsried, Germany

Received, November 14, 2008, and in revised form, June 8, 2009

Published, MCP Papers in Press, July 29, 2009, DOI 10.1074/mcp.M800521-MCP200

© 2009 by The American Society for Biochemistry and Molecular Biology, Inc.
This paper is available on line at http://www.mcponline.org
allows the partial release of InlB into the environment (5, 6). GW domains of soluble InlB interact with glycosaminoglycans (7) and the complement receptor qC1q-R (8) on the host cell surface, although these interactions seem to be dispensable for the process of listerial invasion. In contrast, the N-terminal region of InlB comprising the cap, leucine-rich repeat, and inter-repeat domains (termed InlB$_{321}$) constitutes structural features that stimulate the bacterial ingestion into the host cell cytosol. The horseshoe-like shape of InlB$_{321}$ allows binding to and activation of the transmembrane tyrosine kinase Met, which is also the receptor for the host growth factor, hepatocyte growth factor (HGF). Although InlB binds to a different region of Met compared with HGF, it exploits the Met signaling capabilities, ultimately leading to actin cytoskeleton rearrangements, membrane engulfment, and uptake of the pathogen. InlB induces a rapid autophosphorylation in the kinase domain of Met (9) followed by recruitment of specific adapter molecules initiating signal transduction via prominent downstream components such as PI3K and the Raf-Erk pathway (10). Moreover, immobilized InlB$_{321}$ is sufficient to induce the efficient uptake of latex beads into the host cell (11, 12). Recently, the structure of the InlB$_{321}$-Met complex was solved at the atomic level, unambiguously demonstrating that InlB$_{321}$ is mandatory but also sufficient to activate Met signaling (13).

Numerous molecular studies of signaling components have been reported, and a complex protein network downstream of Met has been compiled (14). However, the molecular interactions defined so far are still insufficient to derive the InlB-induced signal transduction pathway resulting in uptake of Listeria. As a basic signaling principle, protein kinase-catalyzed phosphorylation regulates virtually every function of substrate proteins, i.e. protein-protein interactions, localization, activity, and stability. With more than 500 members, the superfamily of protein kinases is among the largest protein families encoded by the human genome (15). The functional mechanisms regulated by kinase-mediated phosphorylations on substrate proteins are also involved in the activity control of the kinases themselves. Studying these modifications directly at the kinase level enables classification of their activated states, and their systematic investigation by proteomics has already been used to detect and correlate kinases with potential functions in cell cycle control and cancer biology (16, 17). A detailed knowledge of InlB/Met-affected phosphorylation sites of proteins from the kinase superfamily would contribute to a better understanding of the listerial invasion strategy in addition to complementing our knowledge of the Met signaling pathway.

Phosphorylation sites can be detected during the process of automatic peptide sequencing in well established bottom-up proteome approaches. However, the substoichiometric nature and poor ionization properties of phosphopeptides usually require purification strategies such as IMAC to optimize analysis by mass spectrometry (18). Furthermore, the complexity of the total phosphoproteome requires the pre-enrichment of protein kinases as a prerequisite for characterization of the low abundance family members. We and others have demonstrated that the highly conserved ATP-binding region of protein kinases offers possibilities for their systematic purification based on immobilized ATP-competitive small molecule inhibitors with broad kinase selectivity. In combination with phosphopeptide enrichment, this strategy has proven to be highly appropriate for a comprehensive LC-MS/MS-based phosphorylation site analysis of these key signaling components (17, 19, 20). To characterize the role of protein kinases as key regulatory elements in signaling pathways, the acquisition of quantitative peptide data of both the phosphorylated and unmodified proteins is required. Powerful isotopic labeling approaches such as SILAC (21) and iTRAQ™ (22) have been devised and successfully applied to dissect cell and signaling states mainly at the substrate protein level (23, 24), but they are also beginning to support the in-depth characterization of the human kinase (17, 20, 25). Because the detection of individually regulated phosphopeptides has to cope with the so-called “one-hit wonder” problem in proteomics, the interpretation of single peptide regulation requires that particular attention must be paid to the process of statistical raw data evaluation. We have recently established a validated statistical strategy for the quality control of quantitative MS methods used in this study (26). In total, this bioinformatics work flow normalizes unequal sample amounts, corrects isotopic impurities of iTRAQ labeling reagents, and importantly can calculate the reliability of regulatory data based on the actual signal-to-noise properties of the mass spectrometer used.

The synthesis of an optimized affinity resin as a base for a robust single step capture of protein kinases was the starting point in this study and allowed the systematic analyses of this enzyme class in human epithelial cells. In the following, we explain the biochemical strategy established for the quantitative characterization of phosphorylation events at these kinases in the context of infection. The dissection of one representative data set shows the potential of the selected strategy but also underscores the necessity of our statistical approach for evaluating the regulatory information based on iTRAQ reporter ions. Finally, we apply the total approach to analyze protein kinases systematically in the Met receptor kinase pathway exploited by the invasin InlB from L. monocytogenes. The majority of unambiguously regulated phosphorylation events are in accordance with our existing knowledge about the HGF/Met pathway. Furthermore, this study suggests novel candidates such as Nek9 involved in signal networks exploited in the process of listerial invasion.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**

Phospho-Met (Tyr$^{1234/1235}$), phospho-Mek1/2 (Ser$^{217/221}$), phospho-GSK3A/B (Ser$^{37/42}$), phospho-Akt (Ser$^{473}$), and phospho-p44/42 MAPK (Thr$^{202}$/Tyr$^{204}$, Erk1/2) were purchased from Cell Signaling...
Technology Inc., and anti-actin-(20–33) antibody was purchased from Sigma-Aldrich. To prevent phosphatase activity, the following substances were used: calyculin A (LC Laboratories), phosphatase inhibitor mixtures 1 and 2 (Sigma-Aldrich), sodium orthovanadate (Sigma-Aldrich), and sodium fluoride (Merck). The protease inhibitor mixture Complete™ was purchased from Roche Applied Science. Triton X-100 and ethanolamine ReagentPlus were obtained from Sigma-Aldrich, and N,N-dimethylformamide (DMF), triethylammonium bicarbonate, and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC-HCl) were from Fluka. The organic solvents ethanol, methanol, and acetonitrile of pro analysis purity were purchased from J. T. Baker Inc.

**Protein Purification**

InlB321 was recombinantly expressed and purified as described elsewhere (27). In brief, DNA coding for InlB-(36–321) was cloned into the pGEX-6P-1 vector (GE Healthcare) and expressed as a GST fusion protein in *Escherichia coli* BL21 (Stratagen). Cells were disrupted using a French press. Purification of the proteins was performed using Glutathione-Sepharose followed by proteolytic cleavage of the GST tag in solution. Final purification was achieved through Mono Q (GE Healthcare) anion exchange chromatography. Two further InlB321 constructs were expressed and purified as StreptagII fusion proteins (28). In brief, DNA coding for InlB-(36–321) and InlB-(321–36) loss of function (11) were cloned into the pASK-IBA3-Plus vector (IBA GmbH) comprising a tet promoter for controlling InlB expression. Expression was performed in strain E. coli BL21. Recombinatorily expressed InlB was purified from centrifuged French press lysates by Streptactin-Sepharose affinity chromatography and competing elution with desfibrinin. Final purification was again achieved through Mono Q (GE Healthcare) anion exchange chromatography. The recombinant proteins were dialyzed against PBS, concentrated to a final concentration of ~2 mg/ml, filtered through a 0.2-μm membrane, and stored at ~20 °C until use. Stability and purity of the recombinant proteins were investigated by SDS-PAGE. The bioactivity of the recombinantly expressed InlB321 constructs was confirmed in Western blot analyses using phosphorylation site-specific antibodies, indicating the activity state of Met as exemplified in Fig. 1B.

**Western Blot Analysis**

HeLa S3 cells (ATCC CCL 2.2) were grown in DMEM (Invitrogen) supplemented with 2 mM L-glutamine, 10% FBS Gold (PAA Laboratories), 50 units/ml penicillin (Invitrogen), and 50 g/ml streptomycin (Invitrogen) in a 7.5% CO2 humidified 37 °C atmosphere. 4 mM 0.27 M SDS, 0.004% bromphenol blue) containing 1 ml of Benzonase and 1 ml of RNase were added (1 ml/tube) to washed and drained ECH-Sepharose 4B (0.5 ml/tube). The reaction was started by dropwise addition of 1 M EDC-HCl in 50% DMF, EtOH followed by overnight incubation at room temperature in the dark with gentle agitation. After two washes in 50% DMF, EtOH, 1 ml of DMF/EtOH/ethanolamine (1:1:1) was added to block the remaining reactive groups. The reaction was repeated by dropwise addition of 150 μl of 1 mM EDC-HCl in 50% DMF, EtOH followed by overnight incubation at room temperature in the dark with gentle agitation. After two washes in 50% DMF, EtOH, 1 ml of DMF/EtOH/ethanolamine (1:1:1) was added to block the remaining reactive groups. The reaction was repeated by dropwise addition of 150 μl of 1 mM EDC-HCl in 50% DMF, EtOH. Incubation was again conducted at room temperature in the dark overnight with gentle agitation. Sepharose was washed twice with 50% DMF, EtOH; twice with 50 mM HEPES-NaOH, pH 7.5, 0.5 M NaCl; and twice with double distilled H2O. For kinase affinity chromatography four Tricorn 5/50 columns (GE Healthcare) were each packed with 1 ml of VI16743 affinity resin.

**Quantitative Phosphokinome Analysis of the InlB/Met Pathway**

**Preparation of 2-[2-[4-(8-Cyclopentyl-7-oxo-7,8-dihydro-pyrido-[2,3-d]pyrimidin-2-ylamino)-phenoxo]-ethyl]-isoindole-1,3-dione—A solution of 0.32 g (1.00 mmol) of 8-cyclopentyl-2-methanesulfonyl-8H-pyrido[2,3-d]pyrimidin-7-one (29), 0.28 g (1.00 mmol) of 2-[4-(amino-phenoxo)-ethyl]-isoindole-1,3-dione (30), and 0.13 g (0.17 ml, 1.00 mmol) of N-ethylidipropylamine in 20 ml of propan-2-ol was refluxed for 24 h under an argon atmosphere. The solvent was evaporated under reduced pressure, and the residue was partitioned between 30 ml of water and 30 ml of ethyl acetate. The separated water phase was extracted twice with 30 ml of ethyl acetate, the collected organic phase was washed with 30 ml of brine and dried with magnesium sulfate, and the solvent was evaporated under reduced pressure. The residue was crystallized from 10 ml of acetonitrile to give pure product.

**Synthesis of VI16743**

Preparation of 2-[2-[4-(8-Cyclopentyl-7-oxo-7,8-dihydro-pyrido-[2,3-d]pyrimidin-2-ylamino)-phenoxo]-ethyl]-isoindole-1,3-dione and 0.52 g (0.48 ml, 10.00 mmol) of hydrazine hydrate in 25 ml of ethanol was refluxed for 3 h. The solvent was evaporated under reduced pressure, and the residue was partitioned between 30 ml of 3 N HCl in water and 30 ml of ethyl acetate. The separated water phase was extracted four times with 30 ml of ethyl acetate, the pH of the water phase was adjusted to 10 using solid sodium carbonate, and the water phase was extracted three times with 2:1 ethyl acetate:tetrahydrofuran. The organic phase was washed with 30 ml of brine and dried with magnesium sulfate, and the solvent was evaporated under reduced pressure. The residue was crystallized from 5 ml of acetonitrile to give a pure product.
Cell Culture, Treatment, and Lysis

HeLa S3 cells (ATCC CCL 2.2) were grown in DMEM (Invitrogen) supplemented with 2 mM l-glutamine, 10% FBS Gold (PAA Laboratories), 50 units/ml penicillin (Invitrogen), and 50 μg/ml streptomycin (Invitrogen) in a 7.5% CO₂ humidified 37 °C atmosphere. For each quantitative phosphokinome experiment, 1.5 × 10⁶ adherent cells were grown for 24 h on 50 dishes to 50% cell confluence (15-cm diameter, Corning) and then starved for 24 h in DMEM prior to stimulation. In total, four stimulation experiments were performed by treatment of one-half of the cells with 10 nM InlB321 for 4 min. The other half served as control and was either left untreated or incubated with 10 nM InlB321 loss of function, which is unable to bind and activate the receptor tyrosine kinase Met (11). Medium was rapidly discarded after stimulation, 1.5 ml of ice-cold high salt lysis buffer (50 mM HEPES-NaOH, pH 7.5, 1 mM NaCl, 1 mM EGTA, Complete, 10 mM NaF, 2.5 mM Na₃VO₄, 50 ng/ml calyculin A, 1% phosphatase inhibitor mixture 1, 1% phosphatase inhibitor mixture 2, 1% Triton X-100) was added to each dish, and cells were immediately frozen by plunging the bottom of the dishes into liquid nitrogen before storing on dry ice. Samples from all dishes were rethawed in a 4 °C chamber, completely transferred to 50-ml plastic tubes, and solubilized by sonication (5 × 5 s, Bandelin Sonopuls HD200 MS73 lance, Bandelin Electronic). The resulting cell lysates were filtered using a 0.45-μm Sterivex™-HV syringe filter (Millipore) and centrifuged at 70,000 × g for 30 min (4 °C) to remove non-solubilized cell material. The protein concentration of each supernatant was determined by Bradford protein assay (Bio-Rad), and equal protein amounts from InlB-stimulated lysate and from control lysate were used for small molecule kinase affinity chromatography.

Kinase Affinity Chromatography

Two Tricorn 5/50 columns filled with V16743-coupled Sepharose were connected in series for each lysate. Chromatography with both lysates from one experiment was performed in parallel with two GE Healthcare pump P500 systems connected to GE Healthcare valve V7. The columns were equilibrated with buffer A (50 mM HEPES-NaOH, pH 7.5, 1 mM NaCl, 1 mM EGTA, 1 mM EDTA, 10 mM NaF, 0.1 mM Na₃VO₄, 0.1% Triton X-100). Lysates were applied to the columns with a flow rate of 3 ml/h, washed with 60 column volumes of buffer A (6 ml/h), and equilibrated with buffer B (50 mM HEPES-NaOH, pH 7.5, 1 mM EGTA, 1 mM EDTA, 10 mM NaF, 0.1 mM Na₃VO₄, 0.1% Triton X-100). The columns were disconnected, and each column was eluted separately with 0.5% SDS at room temperature (flow rate, 6 ml/h). Twelve 0.5-ml fractions per column were collected. Each fraction was immediately reduced by adding 10% (v/v) 200 mM DTT before incubation at 37 °C overnight. Subsequently, digestion was completed by adding a further 1 μg of trypsin for 2 h to each sample. The peptide solutions were vacuum-dried, and peptides were resolved in 0.2% TFA in water; desalted on self-packed LiChroprep RP-18 (Merck) SPE columns; eluted with 0.2% TFA, 60% ACN in water; and again vacuum-dried.

iTRAQ Modification of Peptides

iTRAQ labeling was performed according to the manufacturer’s protocol (Applied Biosystems). Briefly, dried peptides derived from 120-μg protein fractions of InlB-treated and control cells were resolved in 80 μl of iTRAQ dissolution buffer. To ensure complete iTRAQ labeling two portions of two different labeling reagents, sufficient to label 200 μg of total protein, were dissolved in 70 μl/portions ethanol and used for the labeling reaction that was always conducted for 40 min at room temperature in the dark. Following labeling, both samples from one experiment were combined, vacuum-dried, and desalted on self-packed LiChroprep RP-18 (Merck) SPE columns. The iTRAQ labels used are shown in Table I.

Phosphopeptide Enrichment

A Ga³⁺-based phosphopeptide enrichment kit (Pierce) was used to separate phosphopeptides from non-phosphorylated peptides. All the following steps were carried out in so-called inner surface polymer optimized sample tubes (Roth) to ensure a high recovery of phosphorylated peptides in solution. Combined iTRAQ modified peptides derived from stimulated and control cells were solubilized in 100 μl of binding buffer (1:1:1 methanol-acetonitrile:H₂O containing 2% acetic acid, pH 2.8), and three Ga³⁺-chelated gel disks were added. After 1 h of incubation at room temperature with gentle agitation, unbound peptides were removed by washing 10 times using binding buffer supplemented with 100 μM NaCl followed by washing 10 times with pure binding buffer. Phosphopeptides were specifically eluted with 10 repetitive washes each with 100 μl of 100 mM ammonium phosphate buffer (pH 4.5). The flow-through and all washes were combined and constituted the phosphopeptide-depleted fraction. The IMAC eluate comprising the phosphopeptide-enriched fraction and the phosphopeptide-depleted fraction were vacuum-dried. Both fractions were desalted on self-packed LiChroprep RP-18 (Merck) SPE columns.

Strong Cation Exchange Chromatography, LC-MS/MS Analyses, and Database Searching

The complex phosphopeptide-depleted fraction was further subfractionated by SCX chromatography. Dried peptides were resolved in SCX buffer A (0.065% formic acid, 25% acetonitrile) and separated on a Mono S PC1.6/5 column (GE Healthcare) via an Ettan micro-LC system (GE Healthcare) with a linear gradient from 0 to 35% SCX buffer B (0.065% formic acid, 25% acetonitrile, 0.5 mM potassium chloride) in 30 min. The flow rate was 150 μl/min. 1-minute fractions were collected with a microfraction collector (SunCollect), and peptide elution was monitored at 214 nm. Peptide-containing fractions

| Experiment | Control sample | InlB321-treated sample |
|------------|----------------|------------------------|
| 1          | 114.1          | 117.1                  |
| 2          | 115.1          | 117.1                  |
| 3          | 114.1          | 117.1                  |
| 4          | 115.1          | 117.1                  |
were vacuum-dried and desalted by RP C₁₈ chromatography (μZipTip pipette tips, Millipore).

LC-MS/MS analyses of all desalted SCX fractions and all phosphopeptide-enriched fractions were performed on an Acquity ultra-performance LC system (Waters Corp.) connected to a Q-TOF micro™ mass spectrometer (Waters Corp.). Peptides were flushed onto a C₁₈ precolumn (5-μm Symmetry C₁₈, 180 μm × 20 mm, Waters Corp.) with a flow rate of 15 μl/min and washed at constant flow for 3 min. Peptides were then separated on an analytical column (1.7-μm BEH130, 75 μm × 150 mm, Waters Corp.) with UP LC buffer A (0.1% formic acid in water) and UP LC buffer B (0.1% formic acid in acetonitrile) via linear 90- or 120-min gradients at a flow rate of 300 nL/min controlled with Acquity/UPLC software V1.22. Eluting peptides were ionized using PicoTip emitter needles (New Objective Inc.) and voltages of −1800 kV. Data-dependent acquisition of MS and MS/MS data was under control of MassLynx software V4.1 (Waters Corp.). Dually and triply charged peptide ions were automatically selected and fragmented with m/z-dependent collision energy settings optimized for iTRAQ labeled peptides with a maximum of 18 s per peptide. MS data were automatically processed by MassLynx V4.1, generating peak lists for protein identification by database searches. Database searches were carried out with Mascot Daemon 2.1.6 in the UniProtKB/Swiss-Prot database (release 55.0 of February 26, 2008 with 356,194 entries; taxonomy, Homo sapiens with 18,610 entries). Mascot Daemon was used to merge all peak lists from SCX fractions and IMAC eluate fractions corresponding to one experiment. Proteins were only accepted as identified when at least one unique peptide showed an individual score above 20, which indicated identity or extensive homology (p < 0.05) based on the search parameter settings used (enzyme, trypsin; maximum missed cleavages, 1; fixed modification: carbamidomethyl (Cys), iTRAQ (N terminus), iTRAQ (Lys), variable modifications, phosphorylation (Ser, Thr, Tyr) and oxidation (Met); peptide tolerance, 65 ppm; MS/MS tolerance, 0.15 Da). Phosphopeptides and phosphorylation sites were usually manually confirmed or rejected if the spectrum was of poor quality. Decay searches were performed in a reversed UniProtKB/Swiss-Prot database with search parameters identical to those described above. False discovery rates (FDR) were calculated on the basis of FDR = FP/FP + TP. False positive (FP) and true positive (TP) peptide matches were assigned by counting strictly unique peptide matches (rank 1 and red bold) with confident identification scores (p < 0.05) of target or decoy search results. We determined on average 12% TP, 0.1% FP, and false discovery rates of about 1%. Raw data information of all experiments can be provided on request as a Scaffold data file.

Peptide Quantification

To determine by-product impurities of the iTRAQ reagents a small aliquot from each label was analyzed on a Q-TOF2™ instrument (Micromass, Waters Corp.). Fragmentation was performed by a stepwise increase of collision energy from 10 to 45 eV. The relation of ion intensities of each reporter signal to the detected by-products was calculated from processed spectra. The percentage of impurities was used to determine the sample-specific reporter ion intensities. Peptide quantification was then performed by an in-house bioinformatics tool, named iTRAQassist, as described recently (26). In brief, the Mascot .dat file from a merged peak list from one experiment and the respective by-product calibration file were uploaded. The iTRAQ reporter and regulation base (control) used, as well as the isolation width for the iTRAQ reporter ion detection (0.05 Da) and peptide cutoff (20), were chosen to restrict the quantitative approach to unambiguously identified peptide sequences. iTRAQassist then performed a normalization of reporter ion intensities and provided statistical information for the regulatory data at the individual peptide and protein levels. A documentation of the iTRAQassist functions, the program, and a test data set will be provided on request. Peptides were accepted as regulated if their corresponding likelihood curves showed a distinct separation from the majority of the remaining peptides belonging to the same protein. Finally, all detected regulated peptides were checked manually regarding peptide sequence quality and an unambiguous phosphorylation site assignment. Additionally, regulated candidates were further restricted to peptides that exhibited reporter ion intensities of at least 15 ion counts.

RESULTS

InlB₃₂₁ Is an Effective Activator of Met-mediated Signaling—We used the structurally verified variant of InlB comprising amino acids 36–321 (InlB₃₂₁) to ensure that only Met signaling was observed without cross-activation of co-receptors. InlB₃₂₁ is sufficient to stimulate uptake of coated latex beads, and the recent structure of the Met ectodomain complexed with InlB₃₂₁ revealed how the receptor is locked in a signaling–competent conformation (Fig. 1A). InlB₃₂₁ expression was performed according to Schubert et al. (27), and its capacity to stimulate the Met receptor and its known downstream components was investigated by Western blot analyses. Cells were stimulated for 2, 4, 6, or 8 min by adding extracellular InlB₃₂₁, and subsequently total cell lysates were probed with phosphorylation site-specific antibodies that indicated the activation of Met, Mek1/2, Erk1/2, and Akt and the inhibition of GSK3A/B (pMet, pMek1/2, pErk1/2, pAkt, and pGSK3A/B). Soluble InlB₃₂₁ induced a pronounced autophosphorylation of tyrosines in the kinase domain of Met already within the first 2 min. This activation level of Met did not decrease significantly in the following 6 min. As expected, components of the PI3K/Akt pathway (pAkt and pGSK3A/B) exhibited a rapid phosphorylation site-specific response, confirming their intimate regulatory connection to Met. In contrast, the activation of the Raf-Erk cascade (pMek and pErk) downstream of Met was slightly delayed compared with the PI3K/Akt pathway, and phosphorylation signals were maximally induced 4 min after stimulation with InlB₃₂₁ (Fig. 1B).

Thus, time-resolved analyses established recombantly expressed InlB₃₂₁ as a potent activator of proximal Met signaling. The InlB variant consisting of the leucine-rich repeat structure together with the cap and inter-repeat domains is sufficient to induce Met kinase activity and major Met-dependent downstream cascades. This demonstrates that InlB₃₂₁ is a suitable effector for studying Met-mediated signaling systematically by proteomics. Because all investigated kinases were concomitantly induced after 4 min, we selected this time point for quantitative proteome analysis of the Met pathway.

Humane Kinome Affinity Chromatography Based on Immobilized V16743—Based on the multitarget selectivity of small molecule kinase inhibitors directed against the conserved ATP-binding region, several affinity matrices have been generated that allow the systematic enrichment of protein kinases (20, 33, 34). The immobilized pyridopyrimidine class inhibitor
PP58 was demonstrated as a promising compound for a comprehensive kinase prefractionation strategy from different cell lines (19, 35). However, structural investigations of PP58 in complex with the Src kinase revealed a preference of this inhibitor for protein kinases with small and hydrophilic “gatekeeper” (GK) amino acids at the bottom of the ATP binding pocket (36). Because about 75% of all protein kinases harbor larger side chains at the critical GK position, we modified PP58 to improve its properties as a broad spectrum kinase inhibitor (supplemental Data S1). The resulting molecule, VI16743, was immobilized on Sepharose beads using the free amino group. As previously shown for PP58, this coupling strategy fully retains the kinase binding properties (35).

At first, we determined the kinase spectrum of our novel compound for the systematic analysis of InlB321/Met-dependent protein kinases. Whole cell lysates from the human epithelial cancer cell line HeLa S3 were applied to small molecule affinity chromatography (SMAC) based on immobilized VI16743. Binding, stringent washing, and elution steps were adapted from similar previous kinome analyses, and enriched proteins were identified by peptide sequencing (LC-MS/MS). In total, we could unambiguously identify 409 proteins in the eluted fractions of the VI16743 matrix. Among these, 102 proteins contained a putative kinase domain and were classified as kinases according to the human kinome annotation by Manning et al. (37) and the annotations of the Swiss-Prot database (supplemental Data S2). Although eight of these kinases interact with low molecular weight phosphate acceptors, the majority (94 kinases) transfer phosphate groups to proteins. Importantly, about half of all the unique peptides identified upon affinity purification were derived from protein kinases, indicating high kinase selectivity of immobilized VI16743. Moreover, the combined MS/MS data for the peptides derived from kinases further underscore the specificity of the SMAC strategy: kinases were identified on average by 10 peptides and a median Mascot score of greater than 250, whereas non-kinases were detected on average by only four peptides and a median Mascot score of 86 (Fig. 2A). This demonstrates the specific affinity features of VI16743 for proteins from the superfamily of kinases, which are furthermore substantiated by the high reproducibility of protein kinase identifications: 69% of all kinases were consistently identified in three independent replicate experiments. In contrast, only 37% of non-kinase proteins could be reproducibly identified over the same set of experiments. An alignment of identified protein kinases with the human kinome dendrogram (37) showed that members from virtually all subfamilies of the human kinome were purified by VI16743 affinity chromatography (Fig. 2B) with a slight preference for members of the CMGC (CDK/MAPK/GSK3/CLK family) group. A structural
feature shared among many CMGC group members is the presence of a space-filling phenylalanine at the conserved GK position. Thus, compared with PP58, VI16743 exhibits considerably increased coverage for kinases possessing this structural element. Overall, analyses of GK residues in protein kinases purified by PP58 from three different cell lines indicated a clear preference for the small and hydrophilic threonine (19, 35), whereas VI16743 selected for kinases with the larger and hydrophobic leucine and phenylalanine (supplemental Data S1).

According to estimates that around 300 distinct kinases are expressed in a mammalian cell (38), VI16743 allowed the purification of more than 30% of the human kinome (94 protein kinases) and is presently one of the most effective ATP-competitive ligands for SMAC of protein kinases starting with total cell lysates. Furthermore, VI16743 enabled direct access to Met itself and known downstream kinases such as GSK3A/B, Mek1/2, and Erk1/2 in the context of a systematic screen, suggesting that this resin is an excellent affinity matrix for the direct biochemical investigation of kinase-mediated signal transduction induced by InlB from *Listeria*.

Combination of SMAC, IMAC, iTRAQ, and LC-MS/MS Enables Quantitative Phosphoproteome Analyses of Protein Kinases—Quantitative phosphorylation site analyses of protein kinases can be highly informative in providing details of their activity states within signaling networks. This study addresses the systematic identification of InlB_{321}/Met-dependent phosphorylation events occurring on members of the protein kinase superfamily. All the methods and technologies required are already available in cell biology and proteomics and are now combined to establish a quantitative work flow for phosphokinome analysis (Fig. 3). To reduce potential effects of endogenous HGF, HeLa S3 cells were starved in minimal medium for 24 h. Under these conditions the cells afforded excellent and reproducible responses to external InlB_{321} (Fig. 1B). Subsequently, the cells were stimulated with InlB_{321} for 4 min or left untreated for the same time as a control. iTRAQ labeled phosphorylated and non-phosphorylated peptides from the enriched kinase fractions of stimulated and control cells were finally combined and analyzed by SCX/LC-MS/MS and Mascot. The evaluation of iTRAQ reporter ion information was performed by iTRAQassist (26), an in-house developed software for the calculation and graphical presentation of regulatory events observed at the purified kinases.

Particular attention has to be paid to the systematic and detailed inspection of all regulatory results, starting with the original information from the MS/MS raw data format as exemplified by fragmentation data belonging to the kinases MK01 and Nek9 (Fig. 4). Low energy collisions with argon atoms result in b- and y-ion-dominated fragmentation spectra that facilitate both the peptide sequencing and unambiguous determination of phosphorylation site positions. As recommended by Ross *et al.* (22), we moderately increased the fragmentation energy profiles such that the resulting iTRAQ reporter ions often exhibited intensities similar to those of the y- or b-ion series. Several protein kinases exhibited spectra indicating the induction of phosphorylations following the InlB_{321}-mediated activation of the Met pathway. Fig. 4 displays two such cases where peptides derived from InlB_{321}-treated cells were labeled with iTRAQ-117, whereas the control was labeled with iTRAQ-115. The ratios of 115 and 117 reporter ion signals strongly suggest an InlB_{321}-dependent up-regulation of the depicted phosphorylated peptides. To obtain a general overview of all regulatory events we comparatively analyzed all non-modified peptides and phosphorylated peptides belonging to the same representative experi-
The majority of all MS/MS scans in this experiment suggested regulation factors (RFs) slightly above 1, underscoring the necessity of data normalization. Importantly, the non-modified peptides showed a normal distribution, whereas the phosphorylated peptide fraction revealed a tendency toward larger regulatory events and in addition exhibited a non-normal distribution of regulatory data with some distinct accumulations in the up-regulated region of the total data set. These results indicate that the principal mechanisms of signal transduction, i.e., the dynamic modification of pre-existing signaling components, are also of major relevance in the first steps of the InlB-induced Met pathway. However, the raw data presented in Fig. 5A are still compromised by iTRAQ by-product intensities and did not allow a cumulative view on regulatory peptide data belonging to individual protein kinases. Furthermore, raw data obtained by iTRAQ can vary significantly in quality, depending on the noise characteristics of the MS detector used. Therefore, it is important to account for these factors in a bioinformatics approach for data correction and the quality control of regulatory events. We have recently established a four-step strategy that takes into account all these aspects and finally provides rationalized presentations for evaluation of the reliability of quantitative results (26).
Quantitative Phosphokinome Analysis of the InlB/Met Pathway

A

B

Mascot score: 50.5

Mascot score: 66.8

SS pT333 VTEAPIAVVTSR

M - P - H₂O

1626.0
ties of all MS/MS scans from compared samples are normalized by calculating their trimmed mean. Third, the algorithm computes a weighted cumulated regulation factor for each unique peptide even when it was identified several times.

At this analytical stage the process of iTRAQassist evaluation is still incomplete but allows a first representative view on the investigated protein kinases. Fig. 5B exemplifies the resulting peptide regulation data for the 15 protein kinases that were detected with the highest Mascot identification scores in a typical experiment. This depiction of data allows a cluster analysis of RF values of peptides belonging to the same protein. Most of these RF values from different kinases, such as FAK1, LYN, and GAK, produced noticeable RF clusters with only moderate deviations from an RF value of 1. In addition to these, several peptides exhibited RF values significantly different from the peptides of the main cluster of each protein kinase. Remarkably, and in addition to differentially regulated phosphorylated peptides, several of these...
Quantitative Phosphokinome Analysis of the InlB/Met Pathway

A

Regulation: MK01_HUMAN

![Graph showing phosphorylation sites and likelihoods for MK01_HUMAN regulation.]

Not regulated (significant likelihood)
- GQFVDVGR
- YTNSYIGEGAYGMVCASAYDNVK
- ISPFEHTYQCR
- FRHVISGNNDIIR
- HENIGINDIIR
- APTIEQMK
- DVYIVQDLMETDLKY
- DVYIVQDLMEpDLKY
- THCDSNDHICYFLYQILR
- ICDFGLAR
- NYLSSLPHK
- LFNPADSK
- ALDDLK
- RIEVEQLAHYLEQYDPSDEPIAEAPFK
- LKELIFEETAR
- IEVEQALAHYLEQYDPSDEPIAEAPFK
- ELIFEETAR

Regulated (significant likelihood)
- VADPDHDHTGFLpT184EpY186VATR
- VADPDHDHTGFLT184YEY186VATR

B

Regulation: NEK9_HUMAN

![Graph showing phosphorylation sites and likelihoods for NEK9_HUMAN regulation.]

Not regulated (significant likelihood)
- HCDSINSDFGSESGCDDSSSPGSAQGPR
- AGGGAAEqELHYIPIR
- QVCAGNTFHAVVTVEK
- GAFGEATLYR
- VLAQGLNEFNK
- VASEPHELKPQVEASSPR
- LQQENLQIFTQLOK
- EVYWSWCGEYGR
- QLSFYK
- pS373STVEAPIAVVTQR
- TNLFLTK
- RLGINLLGGPLGGK
- LLTFGCNK
- GWHTILVEK
- RTEDDLSVVWK
- LGDYGLAK

Regulated (significant likelihood)
- SSpT33VTEAPIAVVTSR
peptides were identified as unmodified peptides as detected in CSK22 (CK2α), KPCD2 (PKD2), and AURKB (Aurora B). Because these peptide sequences were identified correctly and represent unique database hits, the data suggest an InlB<sub>321</sub>/Met pathway-dependent posttranslational modification in the corresponding protein regions. As the present study focused on phosphorylation events, we searched for reciprocally regulated phosphopeptides and their unmodified counterparts to support this general assumption. Indeed, we found some pairs representing both the phosphorylated and unmodified peptide species that exhibited reciprocal RF values as expected (supplemental Data S3A). Such cases were also detected for MK01 and GSK3B, confirming that regulated unmodified peptides can coincide with the stimulus-induced regulation of the corresponding phosphorylated amino acid sequence. The MK01 peptide represents a clear InlB<sub>321</sub>-stimulated induction of two phosphorylated residues at Thr<sup>184</sup> and Tyr<sup>186</sup> in a typical activation site motif, TEY, for mitogen-activated protein kinases. The detected up-regulated phosphosite in the kinase GSK3B presented in Fig. 5B corresponds to Ser<sup>9</sup> that mediates kinase inhibition. In conclusion, iTRAQ-detected modifications caused by InlB<sub>321</sub> in the PI3K/Akt pathway and the Raf-Erk cascade compare well with literature data and strongly support the reliability of this phosphoproteomics strategy for studying InlB<sub>321</sub>/Met-dependent phosphorylation events in protein kinases.

However, care must be taken in evaluating RF values. The negative RF value of one unmodified peptide also detected at GSK3B (Fig. 5B) suggests an additional regulation at this protein region, but inspection of the raw data revealed weak and noisy iTRAQ reporter ion signals. Thus, a simple RF value does not allow the evaluation of the robustness of the underlying MS data. Therefore, in a final fourth step, iTRAQassist performs a rational statistical evaluation of the quality of RF values that has been applied to all data sets of this study. iTRAQassist uses a signal-to-noise model algorithm that was established and evaluated previously for the LC-MS work flow used. This model allows prediction of possible variations for any iTRAQ reporter ion intensity and in turn can calculate the robustness of observed RFs at the individual peptide level. The result can be graphically presented as so-called likelihood curves. The top of the curves presents the most likely regulatory factor of individual peptides on the x axis, whereas the intensity on the y axis and the shape of these likelihood curves summarize statistically the underlying quality of the data. Robust regulations will always coincide with good signal-to-noise properties of iTRAQ ions with only a minor probability of variant regulations. Thus, high quality data are presented by small and intense likelihood curves. Contrarily, a flattened curve spanning a larger region of possible RF values refers to uncertainties concerning the actual regulation. In this way, the comparative inspection of likelihood curves belonging to the same protein offers an intuitive way to detect PTM-regulated protein regions as exemplified for MK01 and Nek9 (Fig. 6). This level of iTRAQ data evaluation facilitates the identification of clusters referring to the general expression state of whole proteins. Complementarily, likelihood curves from regulated peptides/phosphorylations will not overlap significantly with these "main clusters" usually observed near RF = 1 in signal transduction experiments.

In the case of MK01 (Fig. 6A), the likelihood view confirms the statistical significance of the regulatory data from the phosphorylated peptides and the corresponding counter-regulated non-modified peptide. The likelihood curve of the detected phosphopeptide DVYIQVQLMEpT<sup>115</sup>DLYK (where pT is phosphothreonine) is depicted as part of the main cluster and, like unmodified peptides, exhibits no specific regulation. In contrast, the doubly phosphorylated peptide VADPDHDHGFLpT<sup>118</sup>EpY<sup>186</sup>VATR (where pY is phosphotyrosine) is up-regulated (RF = 8.9) with a high robustness. Furthermore, alternatively singly and doubly phosphorylated peptides of the same sequence were also detected, but their regulations were of low significance as shown by the flatness of the curves (RF ranging from about 2.5 to 10). The reciprocally regulated, unmodified peptide VADPDHDHGFLTEYVATR (RF = −5.1) also exhibits high robustness, corresponds to all phosphorylated forms with this amino acid sequence, and indicates that a relatively high fraction of MK01 was activated in response to InlB<sub>321</sub>. Applying iTRAQassist and likelihood curve presentations consequently in this study revealed pairs of such reciprocally regulated phosphorylated and non-modified peptides that can be termed significant only at GSK3A, MK01, GSK3B, and CDKL5 (supplemental Data S3B). However, not all phosphorylated peptides might have been identified successfully by the selected IMAC/LC-MS approach, and other types of modifications were not considered in the data search strategy used in this study. Therefore, we also systematically looked for “unpaired” regulated non-modified peptides that may indicate dynamically regulated modifications occurring in these regions of the investigated protein kinases (Fig. 5B and supplemental Data S3C). Actually, a few non-modified regulated peptides with RF values between −2.4 and +8 could be observed in protein kinases, although their likelihood curves overlap partially with curves in the main cluster in most cases (e.g. KPCD2, GSK3B, and PCTK1). Noteworthy are the single peptides from protein kinases CSK22 and M3K3 that are clearly separated from the main cluster, highlighting a significant InlB<sub>321</sub>-induced modification.
Quantitative Phosphokinome Analysis of the InlB/Met Pathway

InlB321-dependent Met activation. Differentially regulated phosphorylation sites following the Western blot analysis (Fig. 1) addition to the InlB321-induced activation site investigated in our previous studies (41–45). Group I comprises phosphorylation sites from all identified proteins that were manually inspected to detect differentially regulated proteins and phosphorylation events in the InlB321/Met pathway. Table II summarizes two groups of protein kinases exhibiting regulation at individual phosphorylation sites following the InlB321-dependent Met activation. Differentially regulated phosphorylation sites of six kinases were already functionally characterized in previous studies (41–45). Group I comprises functionally well-characterized kinases that were already described for the Met pathway and can also serve as proof of concept for this proteome study. In contrast, group II presents a candidate list of regulated kinases as novel Met signaling components that should be evaluated functionally during the process of InlB-mediated bacterial invasion. Because only one time point was investigated by this study, we also accepted kinases as candidates that might have been detected in the earliest or latest stages of dynamic phosphosite regulations. For example, KC1D and Fyn showed moderate but statistically significant (iTRAQassist) regulations of phosphopeptides and might become fully regulated at other time points (see likelihood plots in supplemental Data S5). In addition to the InlB321-induced activation site investigated in our Western blot analysis (Fig. 1B), we detected a regulated phosphopeptide mapping to the outer C-terminal region of MP2K2 (Mek2), which belongs to the MAPK pathway. KS6A3, a so-called MAPK-activated protein kinase downstream of the MAPK cascade, was also found to harbor a differentially phosphorylated peptide. Manual inspection of the MS/MS data revealed that the modification might be located either at Thr736 or at Ser737. Thus, the systematic characterization of protein kinases by the presented proteomics approach shows that InlB321 is also a potent activator of the Met-dependent MAPK and the PI3K pathways similar to HGF. Furthermore, our data suggest a negative phosphorylation site regulation in response to InlB321 stimulation, namely Thr208 (corresponding to MARK2) in the activation loop of microtubule-associated protein/microtubule affinity-regulating kinase (MARK) kinase family members. We detected unique peptides from MARK1, MARK2, and MARK3 but not from MARK4, which is therefore most likely not expressed in HeLa S3 cells. Further regulated phosphorylation sites were detected on CDK5 and AMPK. Interestingly, AMPK is involved in actin cytoskeleton dynamics, a process that is certainly contributing to the uptake of L. monocytogenes into the cell. However, the most robust and pronounced response was observed at Nek9 among all novel candidates. Its InlB321-induced regulation at position 333 (Thr333) could be identified unambiguously and provides a biochemical basis for functional investigations of this protein kinase. The modified residue is located close to a nuclear localization signal of Nek9, favoring a direct influence on the cellular localization of this kinase in response to Met activation.

DISCUSSION

Protein kinases are key components involved in the control of virtually every signaling cascade. Kinase-mediated phosphorylation tightly regulates the activity, localization, and stability as well as the molecular interactions of substrate proteins. Thus, the systematic characterization of dynamic phosphorylation events is currently one primary goal in infection research as pathogens exploit and manipulate host signaling by effectors to realize individual steps of their infection cycle. InlB from L. monocytogenes mediates invasion by binding and activation of the host receptor kinase Met, but both the “mode of action” and the details of possible effects of the released form of InlB on neighboring cells and tissues remain unknown. Interestingly, neither the well-characterized Met pathway nor the structural investigation of the InlB321-Met complex provides intuitive concepts to explain the induced uptake of *Listeria* into the cells. The InlB-Met interaction might therefore constitute novel signaling mechanisms. Study of the InlB-induced proximal signaling will most likely complement our understanding, and this is probably most easily accessed using chemical proteomics that already has strategies for the systematic analyses of human protein kinases. Efficient enrichment of kinases can be achieved by immobilized kinase inhibitors, allowing their affinity purification from highly com-
plex protein samples and greatly facilitating the comprehensive analysis of posttranslationally modified forms by LC-MS/MS approaches. VI16743 used in this study is to our knowledge one of the most nonspecific ATP-competitive protein kinase inhibitors and by far exceeds the capture efficiency of the previously described PP58 based on the same chemical scaffold. In earlier studies, PP58-based affinity purifications permitted the identification of 84 distinct protein kinases from three different cell lines of which each was analyzed using 5*10^9 cells as starting material (19, 35). In contrast, VI16743 chromatography in combination with similar LC-MS instrumentation identified a total of 94 protein kinases derived from virtually every branch of the human kinome, although only one cell line and 10 times less total cell extract served as starting point in this study.

Whereas the identification of phosphorylation sites on kinases can already provide important clues for the understanding of disease-related processes, the detection of regulated phosphorylation events is particularly beneficial to reveal components participating in signal transduction or certain cellular processes (17, 20). In the present work, we combined an LC-MS/MS-based strategy for systematic phosphorylation site determination in the human kinome with a survey of quantitative data using iTRAQ peptide labeling. Several statistical methods for iTRAQ data evaluation have been presented previously (46–48), but these were mainly focused on the quantification of protein expression and not on PTM regulation. Therefore, and driven by the challenge to establish a proteome-based work flow for characterization of pathogen-induced host signaling pathways, we have recently described a rational statistical approach to unambiguously detect significantly regulated modifications occurring at individual peptides from one protein (26). In total, we investigated 143 unique phosphorylation sites at serine, threonine, and tyrosine with a ratio of 64:28:8. Thus, threonine and in particular tyrosine phosphorylation are highly over-represented regulatory

### Table II

| Protein kinase | Regulated phosphorylation site | Site regulation | Unmodified peptide with reverse RF | Site location | Function of phosphorylation |
|---------------|-------------------------------|----------------|---------------------------------|--------------|-----------------------------|
| MK03 (Erk1)  | pT202pY204                   | 18 +++         | no                              |              | Activation (Mckay, 2007)    |
| MK01 (Erk2)  | pT185pY186                   | 31 +++         | yes                             |              | Activation (Mckay, 2007)    |
| MP2K2 (Mek2) | pT396                        | 6 +            | no                              |              | unknown                      |
| GSK3A        | pS21                         | 4 +++          | yes                             |              | Inhibition (Srivastava, 1998) |
| GSK3B        | pS9                          | 3 +++          | yes                             |              | Inhibition (Srivastava, 1998) |
| NEK9         | pT133                        | 4 +++          | no                              |              | unknown                      |
| AMPK1 (AMPKε1)| pS407                       | 4 ++           | unknown                         |              | unknown                      |
| CDKL5        | pS407                        | 3 ++           | yes                             |              | unknown                      |
| K56A3 (RSK2) | pT730 or pS737               | 5 +            | unknown                         |              | Activation state control     |
| MARK2        | pT200                        | -2 +           | no                              |              | Activation (Timm, 2006)     |
| FYN          | pY620 (S)                    | 1.5 +          | no                              |              | Molecular association (Nguyen, 2002) |
| KC1D (CK1D)  | pS82                         | -1.7 +         | no                              |              | unknown                      |

RF: Regulation Factor; a: according to iTRAQassist (26) and manual inspection
§: Peptide sequence homologues in FK [SRC] [LCX] YES

**Quantitative Phosphokinome Analysis of the InlB/Met Pathway**
Quantitative Phosphokinome Analysis of the InlB/Met Pathway

modifications for kinases compared with their contribution to total cellular phosphorylation where a ratio of 90:10:0.5 was found (49).

Quantitative peptide sequencing in combination with iTRAQassist revealed 12 InlB$_{321}$-induced regulated phosphorylation sites in different protein kinases (Table II). Furthermore, we observed a few unmodified peptides that show InlB$_{321}$-dependent regulation factors, whereas the majority of unmodified peptides from the same protein kinase were not regulated (see MK01 in Fig. 6A). Daub et al. (17) recently showed that such non-modified peptide regulations can represent “counter-regulations” that coincide with the parallel increase or decrease of phosphorylations occurring in the same protein region. However, the present study could only reveal four pairs of phosphorylated and non-modified peptides of the same sequence that are significantly counter-regulated (supplemental Data S3C). Hence, similar approaches will most likely facilitate the identification of protein regions regulated by PTMs other than phosphorylation. However, their success will depend on protein coverages achieved by bottom-up proteomics and the ratio of proteins actually participating in the process of modification/signal transduction. Actually, several non-modified peptide regulations seem to behave differently from the rest of the protein, but unfortunately these regulations often could not be termed significant in comparison with other peptides of the main cluster nor could this study identify the corresponding modified peptides (supplemental Data S3C).

Focusing on phosphosite regulation, our approach identified known Met signaling events, thus substantiating the relevance of quantitative proteome data for signal transduction studies. More importantly, this study also revealed a new subset of Met-dependent protein kinases potentially exploited by InlB from the pathogen L. monocytogenes. Interestingly, several of these kinase functions can either be related to known HGF/Met-controlled cellular phenotypes or even be linked to the process of bacterial invasion (summarized in Fig. 7). One such promising candidate is the family of MARK kinases that controls the stability of microtubule (MT) filaments. Microtubule-destabilizing agents such as nocodazole strongly reduce invasion efficiency of L. monocytogenes into different cell lines (50–52). The maintaining of MT filaments therefore might be a feasible strategy of the pathogen to support the first steps of the infection cycle. A possible connection to cytoskeleton dynamics was also recently identified for the protein kinase AMPK that we found to be regulated. Besides its known function as an energy sensor (53), AMPK appears to be involved in actin cytoskeleton dynamics, cell polarity, and cell cycle-related processes (54, 55). We identified an up-regulation of Ser$^{487}$ in the outer C-terminal region of the catalytic subunit of the heterotrimeric AMPK complex. As yet, the role of AMPK in the InlB-activated Met pathway has not been characterized. Its possible influence on the actin cytoskeleton might also contribute to listerial invasion efficiency. This is supported by the fact that Akt1, as a component of the PI3K/Akt1 pathway, contributes to the InlB/Met-dependent process of invasion (56, 57) and was identified as being responsible for the phosphorylation of the catalytic AMPK subunit at Ser$^{487}$ (58, 59). Other kinases such as CDK5 have poorly characterized functions, and no information is available about the identified regulated phosphorylation sites. iTRAQassist also indicated robust regulations at well characterized kinases such as Fyn and casein kinase 1. It was demonstrated that HGF induces the increased association of Fyn with Cbl, which mediates ubiquitination and consequently uptake and degradation of the Met receptor (60), also an indispensable process for Listeria invasion (14). Casein kinase 1 can also be linked to natural Met-dependent responses because recent studies substantiate its regulatory role in cell cycle progression and mitosis (61). However, time-resolved phosphorylation studies at both kinases must first verify the hypothesis that the observed weak regulations actually indicate a more intensive regulation at other time points.

Besides the already known Met signaling components, the most pronounced and robustly regulated phosphorylation site was detected at the cell cycle-related kinase Nek9. Phosphorylation at Thr$^{333}$ was strongly up-regulated after 4-min treatment with InlB$_{321}$. This modification site is located adjacent to a functional nuclear localization signal downstream of the kinase domain (62). Nek9 is described as a contributor to cell cycle progression (63) as well as DNA transcription (64), and a nuclear-cytoplasmic distribution was observed (63). The modification of Thr$^{333}$ in the Met pathway might disrupt a supposed intramolecular loop (62), thus exposing the nuclear localization signal and resulting in nuclear shuttling of Nek9. This modification might prime Nek9 for dimerization, which is essential for autophosphorylation at Thr$^{110}$ and correlates with kinase activation. Whereas an evaluation of all presented candidates by immunological methods should confirm the presented proteomics data, the functional investigation of Nek9 is already now obligatory based on the existing data quality. Prospective functional approaches (e.g. by RNA interference) will help to understand the individual contributions of protein kinases with respect to listerial invasion and physiological cell responses to Met activation. In addition, site-directed mutations of regulated phosphorylation sites should allow an evaluation of the influence of the identified phosphorylation sites in the InlB-Met system, thus gaining new insights into the function of these partially uncharacterized protein kinases.

Infection research has focused in the past on the detection and functional characterization of virulence factors from different human pathogens. Among these, numerous effectors have been described that manipulate the host and realize individual steps in the infection cycle (65). Interestingly, successful screenings for the host-interacting proteins and even structural studies do not necessarily explain effector-induced processes mechanistically. Internalin B from L. monocytogenes obviously exploits the Met signaling pathway to invade different cell types, but the signaling network involved that coordinates such a process has
FIG. 7. Met signal transduction exploited by InlB from *L. monocytogenes*. InlB<sub>321</sub>-dependent differentially phosphorylated protein kinases identified in the present study are highlighted in blue. *L. monocytogenes* activates the PI3K and MAPK pathways, both essential for actin cytoskeleton remodeling as a prerequisite for invasion. The identified induction of phosphorylation events on RSK2 and MEK support a negative feedback theory in this signaling module. Dephosphorylation of MARK2 in the kinase domain as one novel finding is suggested to block MARK2 activation, which is probably essential for *Listeria* invasion. Active MARK2 leads to destabilization of MTs by phosphorylation of microtubule-associated proteins. Earlier studies already demonstrated that destabilization of MTs by nocodazole impaired efficient *Listeria* uptake by the host cell. The functional contribution of novel candidates such as Nek9 or CK1D to the InlB/Met invasion strategy will be the subject of further studies. *Ub*, ubiquitin.
not been characterized in detail to date. This study presents a quantitative proteomics strategy that focuses on protein kinases that form an essential part of the host signaling network. The results of this study demonstrate modifications at known and potentially novel InlB/Met pathway components, providing a robust resource for Listeria research. Furthermore, the InlB-affected proximal host signaling raises questions whether these kinases might be involved in the natural physiological function of the Met pathway. It will be obligatory now to attempt the direct comparison of InlB versus HGF signaling, and time-resolved experiments will further facilitate the compilation of hierarchical protein kinase cascades. This information may define possible intervention points for preventing listerial invasions in immunocompromised patients and certainly will extend our knowledge of this fundamentally important host signaling pathway that is frequently found deregulated in several types of cancer.

Acknowledgments—We thank Kirsten Minkhart and Reiner Munder for technical assistance, Dr. Uwe Käst for fruitful discussions, and Dr. Victor Wray for proofreading the manuscript. We thank Axel Ulrich for support of this study with funding from the Department of Molecular Biology, Max Planck Institute of Biochemistry.

* This work was supported by European Research Area ERAnet Project 0313939B (SPATELIS, Spatio-temporal analysis of Listeria-host protein interactions) and by funding from the Department of Molecular Biology, Max Planck Institute of Biochemistry. The on-line version of this article (available at http://www.mcponline.org) contains supplemental Data S1–S5.

** To whom correspondence should be addressed. E-mail: lothar.jansch@helmholtz-hzi.de.

REFERENCES

1. Lecuit, M., Vandommael-Pourain, S., Lefort, J., Huerre, M., Gounon, P., Dupuy, C., Babinet, C., and Cossart, P. (2001) A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. Science 292, 1722–1725

2. Mengaud, J., Ohayon, H., Gounon, P., Mege, R.-M., and Cossart, P. (1996) E-cadherin is the receptor for internalin, a surface protein required for entry of L. monocytogenes into epithelial cells. Cell 84, 923–932

3. Drum, S., Biswas, I., Maguin, E., Braun, L., Mastroeni, P., and Cossart, P. (1995) Entry of Listeria monocytogenes into hepatocytes requires expression of inlB, a surface protein of the internalin multi gene family. Mol. Microbiol. 16, 251–261

4. Parida, S. K., Domann, E., Rohde, M., Muller, S., Darji, A., Hain, T., Wehland, J., and Chakraborty, T. (1998) Internalin B is essential for adhesion and mediates the invasion of Listeria monocytogenes into human endothelial cells. Mol. Microbiol. 28, 81–93

5. Trost, M., Wehmhoener, D., Kast, U., Dietrich, G., Wehland, J., and Jansch, L. (2005) Comparative proteome analysis of secretory proteins from pathogenic and nonpathogenic Listeria species. Proteomics 5, 1544–1557

6. Jonqueres, B., Bieger, H., Fiedler, F., Gounon, P., and Cossart, P. (1999) Interaction between the protein inlB of Listeria monocytogenes and lipoteichic acid: a novel mechanism of protein association at the surface of gram-positive bacteria. Mol. Microbiol. 34, 902–914

7. Jonqueres, B., Pizarro-Cerdà, J., and Cossart, P. (2001) Synergy between the N- and C-terminal domains of inlB for efficient invasion of non-phagocytic cells by Listeria monocytogenes. Mol. Microbiol. 42, 955–965

8. Marino, M., Banerjee, M., Jonqueres, B., Cossart, P., and Ghosh, P. (2002) GW domains of the Listeria monocytogenes invasion protein InlB are SH3-like and mediate binding to host ligands. EMBO J. 21, 5623–5634

9. Shen, Y., Naujokas, M., Park, M., and Iretan, K. (2000) InlB-dependent internalization of Listeria is mediated by the Met receptor tyrosine kinase. Cell 103, 501–510

10. Copp, J., Marino, M., Banerjee, M., Ghosh, P., and van der Geer, P. (2003) Multiple regions of internalin B contribute to its ability to turn on the Ras-mitogen-activated protein kinase pathway. J. Biol. Chem. 278, 7783–7789

11. Machner, M. P., Frese, S., Schubert, W. D., Orian-Rousseau, V., Gherardi, E., Wehland, J., Niemann, H. H., and Heinz, D. W. (2003) Aromatic amino acids at the surface of InlB are essential for host cell invasion by Listeria monocytogenes. Mol. Microbiol. 48, 1525–1536

12. Braun, L., Nato, F., Payrastre, B., Maziec, J. C., and Cossart, P. (1999) The 213-amino-acid leucine-rich repeat region of the listeria monocytogenes InlB protein is sufficient for entry into mammalian cells, stimulation of PI-3-kinase and membrane ruffling. Mol. Microbiol. 34, 10–23

13. Niemann, H. H., Jager, V., Butler, P. J., van den Heuvel, J., Schmidt, S., Ferraris, D., Gherardi, E., and Heinz, D. W. (2007) Structure of the human receptor tyrosine kinase met in complex with the Listeria invasion protein inlB. Cell 130, 235–246

14. Hannon, M., Bierne, H., and Cossart, P. (2006) Listeria monocytogenes is a multifaceted model. Nat. Rev. Microbiol. 4, 423–434

15. Mann, G. (2005) Genomic overview of protein kinase expression. WormBook 1–19

16. Rikova, K., Guo, A., Zeng, Q., Possemato, A., Yu, J., Haack, H., Nardone, J., Lee, K., Reeves, C., Li, Y., Hu, Y., Tan, Z., Stokes, M., Sullivan, L., Mitchell, J., Wetzl, R., Macneill, J., Ren, J. M., Yuan, J., Bakalariski, C. E., Villen, J., Kornhauser, J. M., Smith, B., Li, D., Zhou, X., Gygi, S. P., Gu, T. L., Polakiewicz, R. D., Rush, J., and Comb, M. J. (2007) Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. Cell 131, 1190–1203

17. Daub, H., Olsen, J. V., Barik, M., Gnad, F., Oppermann, F. S., Körner, R., Greff, Z., Kéri, G., Stemmann, O., and Mann, M. (2008) Kinase-selective enrichment enables quantitative phosphoproteomics of the kinome across the cell cycle. Mol. Cell 31, 438–448

18. Corthals, G. L., Aebersold, R., and Goodlett, D. R. (2005) Identification of phosphorylation sites using microimmobilized metal affinity chromatography. Methods Enzymol. 405, 66–81

19. Wehland, J., Jansch, L., Nimtz, M., Dietrich, G., Hornberger, R., Kéri, G., Wehland, J., and Daub, H. (2007) Proteomics analysis of protein kinases by target class-selective prefractionation and tandem mass spectrometry. Mol. Cell Proteomics 6, 537–547

20. Bantscheff, M., Eberhard, D., Abraham, Y., Bastuck, S., Boesche, M., Hübner, S., Mathieson, T., Perrin, J., Raida, M., Rau, C., Reader, V., Sweetman, G., Bauer, A., Buermester, T., Hopf, C., Kruse, U., Neubauer, G., Ramsden, N., Rick, J., Kuster, B., and Drewes, G. (2007) Quantitative chemical proteomics reveals mechanisms of action of clinical ABL kinase inhibitors. Nat. Biotechnol. 25, 1035–1044

21. Ong, S. E., Blagoev, B., Kratchmarova, I., Kristensen, D. B., Steen, H., Pandey, A., and Mann, M. (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol. Cell Proteomics 1, 376–386

22. Daub, H., Olsen, J. V., Huang, Y. N., Marches, J., Williams, B. R., Park, K., Hattan, S., Khainovskii, N., Pillai, S., Dey, S., Daniels, S., Purkayastha, S., Juhasz, P., Martin, S., Bartlet-Jones, M., He, F., Jacobson, A., and Pappin, D. J. (2004) Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol. Cell Proteomics 3, 1154–1169

23. Olsen, J. V., Blagoev, B., Gnad, F., Macek, B., Kumar, C., Mortensen, P., and Mann, M. (2006) Global, in vivo, and site-specific phosphorylation dynamics. Cell 127, 635–648

24. Chen, Y., Choong, L. Y., Lin, Q., Philp, R., Wong, C. H., Ang, B. K., Tan, Y. L., Loh, M. C., Hew, C. L., Shah, N., Druker, B. J., Chong, P. K., and Lim, Y. P. (2007) Differential expression of novel tyrosine kinase substrates during breast cancer development. Mol. Cell Proteomics 6, 2072–2087

25. Bantscheff, M., Boesche, M., Eberhard, D., Matthieson, T., Sweetman, G., and Kuster, B. (2008) Robust and sensitive iTRAQ quantification on an LTQ Orbitrap mass spectrometer. Mol. Cell. Proteomics 7, 1702–1713

26. Hertedtmarker, C., Fischer, R., Reini, T., May, S., Klawonn, F., and Jänisch, L. (2009) MS-specific noise model reveals the potential of iTRAQ™ in quantitative proteomics. Bioinformatics 25, 1004–1011

27. Schubert, W. D., Göbel, G., Diepolz, M., Darji, A., Kloer, D., Hain, T., Chakraborty, T., Wehland, J., Domann, E., and Heinz, D. W. (2001)
