Src-dependent Tyrosine Phosphorylation of Non-muscle Myosin Heavy Chain-IIA Restricts Listeria monocytogenes Cellular Infection*

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Background: Non-muscle myosin II is involved in force generation, movement, and membrane reshaping. Its activity is regulated by phosphorylation of the light chain.

Results: NMHC-IIA head domain is tyrosine-phosphorylated by Src and modulates Listeria intracellular levels.

Conclusion: Tyrosine phosphorylation of NMHC-IIA affects the outcome of infection.

Significance: This novel post-translational modification of NMHC-IIA possibly affects its functions.

Bacterial pathogens often interfere with host tyrosine phosphorylation cascades to control host responses and cause infection. Given the role of tyrosine phosphorylation events in different human infections and our previous results showing the activation of the tyrosine kinase Src upon incubation of cells with Listeria monocytogenes, we searched for novel host proteins undergoing tyrosine phosphorylation upon L. monocytogenes infection. We identify the heavy chain of the non-muscle myosin II (NMHC-IIA) as being phosphorylated in a specific tyrosine residue in response to L. monocytogenes infection. We characterize this novel post-translational modification event and show that, upon L. monocytogenes infection, Src phosphorylates NMHC-IIA in a previously uncharacterized tyrosine residue (Tyr-158) located in its motor domain near the ATP-binding site. In addition, we found that other intracellular and extracellular bacterial pathogens trigger NMHC-IIA tyrosine phosphorylation. We demonstrate that NMHC-IIA limits intracellular levels of L. monocytogenes, and this is dependent on the phosphorylation of Tyr-158. Our data suggest a novel mechanism of regulation of NMHC-IIA activity relying on the phosphorylation of Tyr-158 by Src.

Listeria monocytogenes is a human intracellular food-borne bacterial pathogen that causes serious disease in immunocompromised individuals. Within the host it finds suitable replication niches in the liver and spleen, disseminates, and then can reach the central nervous system. In pregnant women, L. monocytogenes targets the fetus, eliciting fetal infection and abortions (1). The ability of L. monocytogenes to cause disease relies on its capacity to invade nonphagocytic cells, replicate therein, and spread to the entire organism overcoming the intestinal, blood-brain, and fetoplacental barriers (2). Through the expression of bacterial factors, L. monocytogenes establishes a cross-talk with host cells favoring the progression of the cellular infection (3). In epithelial cells, L. monocytogenes invasion is mainly driven by the bacterial surface proteins InIA and InIB that bind E-cadherin and c-Met, respectively, at the surface of host cells (4, 5). This engagement of host cell receptors triggers tyrosine phosphorylation-mediated signaling, resulting in the local activation of the Arp2/3 complex that initiates actin polymerization at the site of L. monocytogenes attachment (6, 7), causing membrane invagination that supports bacterial entry. InIB interaction with the receptor tyrosine kinase c-Met stimulates its auto-phosphorylation and induces the tyrosine phosphorylation and recruitment of adaptor proteins and the activation of phospho-
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inositide 3-kinase (PI3K) (5, 8, 9). Phosphatidylinositol 3,4,5-triphosphate generated by PI3K accumulates at the cell membrane during L. monocytogenes infection (8) and plays a crucial role in the recruitment of molecules controlling actin polymerization, such as Rac1 and WAVE2 (6, 10–12). In turn, InLA binding to E-cadherin induces the activation of Src tyrosine kinase that subsequently phosphorylates cortactin, E-cadherin, and the clathrin heavy chain (7, 13, 14). Although cortactin and clathrin tyrosine phosphorylations are critical events for actin polymerization and recruitment at the L. monocytogenes entry site (7, 13), E-cadherin phosphorylation leads to its ubiquitination, internalization, and further degradation (14). The combined action of these events leads to the internalization of the L. monocytogenes into epithelial cells.

In this study we aimed to identify new cellular proteins undergoing tyrosine phosphorylation in response to L. monocytogenes infection, and we address whether such post-translational modification would regulate cellular infection. The tyrosine-phosphorylated proteins were recovered from L. monocytogenes-infected epithelial cells and subjected to mass spectrometry identification. We identified the non-muscle myosin heavy chain IIA (NMHC-IIA)6 as one of the enriched tyrosine-phosphorylated proteins recovered upon L. monocytogenes infection.

NMHC-IIA is an actin-binding protein with motor and contractile properties, involved in cellular processes requiring force generation, cell movement, and membrane reshaping (15). In infection, NMHC-IIA is critical for viral entry (16, 17) and supports invasion (18) and dissemination (19) of various bacteria. Although the serine/threonine phosphorylation of the regulatory light chain is a well known mechanism to regulate non-muscle myosin IIA activity (15), our knowledge on the regulation of the heavy chain is limited, and NMHC-IIA tyrosine phosphorylation has never been characterized. Here, we show that NMHC-IIA undergoes tyrosine phosphorylation in response to several bacterial pathogens. Our data indicate that upon L. monocytogenes cellular infection NMHC-IIA was phosphorylated in tyrosine residue 158 by the host Src kinase. In the presence of blebbistatin, a chemical inhibitor of myosin II activity, the percentage of cells showing L. monocytogenes-associated actin foci was increased and correlated with higher levels of intracellular L. monocytogenes. In addition, increased numbers of intracellular L. monocytogenes were also found in cells depleted of NMHC-IIA as well as in conditions where NMHC-IIA tyrosine phosphorylation is prevented. These results show the involvement of NMHC-IIA in L. monocytogenes infection and point to the regulatory role of its phosphorylation in tyrosine 158, which could affect NMHC-IIA activity. Our findings describe a novel post-translational modification of NMHC-IIA with important implications in bacterial infection. Taking into account the central role of NMHC-IIA in key cell biology processes, our data also suggest the existence of a new mechanism of NMHC-IIA regulation that could be of critical importance in the canonical functions of non-muscle myosin IIA.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Cell Lines—Listeria and Escherichia coli strains were grown aerobically at 37 °C, with shaking, in brain-heart infusion and lysogeny broth (LB) media, respectively. Yersinia was grown aerobically at 26 °C, with shaking, in LB media. When required, antibiotics were added to growth media. Details are provided in Table 1. Caco-2 cells (ATCC HTB-37) were cultivated in minimum Eagle’s medium with 10% fetal bovine serum (FBS). HeLa (ATCC CCL-2), HEK293 (ATCC CRL-1573), and COS-7 (ATCC CRL-1651) cells were cultivated in DMEM with glucose (4.5 g/liter) and fetal bovine serum (FBS). Cell culture media and supplements were from Lonza.

Plasmids, Antibodies, and Reagents—Plasmids used are listed in Table 2. Plasmids GFP-NMHC-IIA-Y158F and GFP-NMHC-IIA-Y190F were generated using GFP-NMHC-IIA-WT from Addgene (20) and the QuikChange XL site-directed mutagenesis kit (Agilent Technologies). For NMHC-IIA rescue assays, a plasmid encoding siRNA-resistant GFP-NMHC-IIA-WT transcripts was generated. Oligonucleotide sequences are provided in Table 3. Antibodies are listed in Table 4. F-actin was labeled with Alexa Fluor 647- or 555-conjugated phalloidin (Invitrogen). Chemical inhibitors Y-26732 (Sigma), blebbistatin, and PP1 (Calbiochem) were handled as recommended. FluorSpheres carboxylate-modified microspheres were from Invitrogen (F-8814).

| Bacterial strains | Growth media | T  |
|-------------------|--------------|----|
| L. monocytogenes (EGDe) | BHI (Difco) | 37 |
| L. innocua (CLIP 11262) | BHI | 37 |
| L. innocua-inlB | BHI erythromycin 5 μg/ml | 37 |
| E. coli DH5α | LB (Difco) | 37 |
| EPEC | LB ampicillin 100 μg/ml (Amp100) | 37 |
| EHEC | LB | 37 |
| E. coli K12-inv | LB Amp100 | 37 |
| E. coli K12-Δinv | LB Amp100 | 37 |
| Y. pseudotuberculosis | LB Amp100 | 26 |

The abbreviations used are: NMHC-IIA, non-muscle myosin heavy chain IIA; NMHC-IIB, non-muscle myosin heavy chain IIb; WCL, whole cell lysate; m.o.i., multiplicity of infection; NI, noninfected; IP, immunoprecipitation; NT, nontreated; Ctr, control; MLCK, myosin light chain kinase; EPEC, enteropathogenic E. coli; EHEC, enterohemorrhagic E. coli; KSHV, Kaposi sarcoma-associated herpesvirus.

Determination of Intracellular Bacteria—The levels of intracellular bacteria were determined as described (21). When indicated, cells were incubated with serum-free medium containing blebbistatin, PP1, or DMSO. Cells were challenged with prewashed L. monocytogenes at a multiplicity of infection (m.o.i.) of 50 or with Yersinia pseudotuberculosis (m.o.i. 10) for 60 min, treated with 20 μg/ml gentamicin for 90 min, washed in PBS, and lysed with 0.2% Triton X-100, and serial dilutions were plated for CFU counting. For immunofluorescence scoring, cells infected with L. monocytogenes (m.o.i. 50) were treated with 100 μg/ml gentamicin for 10 min and washed with 20 μg/ml gentamicin prior fixation.

Immunoprecipitation Assays—HeLa or Caco-2 cells grown until confluence were washed twice with warm phosphate-
buffered saline (PBS), serum-starved (5 h), and left noninfected (NI) or incubated with pre-washed *L. monocytogenes* (21) at m.o.i. 200 for different periods of time or with *E. coli* (EPEC, EHEC, or K12-*inv* strains) at m.o.i. 200 for 4 h as described (22). When indicated, cells were treated with 10 μM PP1 or 50 μM Y-27632 30 min before infection. After washing twice with ice-
cold PBS, cells were lysed in 1 ml of lysis buffer (1% IgePal CA-630 (Sigma), 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (Interchim), PhosSTOP, and eComplete Protease Inhibitor Mixture (Roche Applied Science)), and lysates were recovered after centrifugation (15,000 × g, 15 min, 4 °C). Cell lysates (500 μg) were incubated overnight (4 °C) with 1 μg of anti-phosphotyrosine 4G10 or 5 μg of anti-NMHC-IIA antibodies. Immune complexes were captured with 50 μl of Pure Proteome protein A or G magnetic beads (Millipore). Immunoprecipitated fractions were resolved by SDS-PAGE, and gels were silver-stained using the ProteoSilver™ plus silver staining kit (Sigma) or processed in 0.2M glycine, pH 2.5.

Immunofluorescence Analysis—Cells were fixed in 3% para-formaldehyde (15 min), quenched with 20 mM NH₄Cl (1 h), permeabilized with 0.1% Triton X-100 (5 min), and blocked with 1% BSA in PBS (30 min). Antibodies were diluted in PBS containing 1% BSA. Coverslips were incubated for 1 h with primary antibodies washed three times in PBS and incubated 45 min with secondary antibodies and phalloidin Alexa 555 or 647. DNA was counterstained with DAPI (Sigma). Coverslips were mounted onto microscope slides with Aqua-Poly/Mount (18606, Polysciences). Images were collected with a confocal laser-scanning microscope (Zeiss Axiosvert LSM 510 or Leica SP2 AOBS S.E.) and processed using Adobe Photoshop software.

Transfection and Lentiviral Transduction—The lentiviral shRNA expression plasmids Mission pLKO.1-puro (control) and Mission shRNA-c-Src (Sigma) were used in combination with the envelope plasmid pMD.G and packaging plasmid pCMV/R8.91. Packaging, envelope, and shRNA vector plasmids were co-transfected into HEK293 cells. Viral supernatants were harvested after 72 h, filtered, and incubated with target HeLa cells for 48 h at 37 °C. Puromycin was used to select for individual clones. The knockdown was verified by immunoblot and/or real time RT-PCR.

Protein Identification by Mass Spectrometry (MS)—Protein identification was performed by MALDI TOF/TOF mass spectrometry as described (23). Protein bands were excised from SDS-polyacrylamide gels, reduced with dithiothreitol, alkylated with iodoacetamide, and in-gel digested with trypsin. Peptides were extracted, desalted, concentrated using Ziptips (Millipore), crystallized onto a MALDI sample plate, and analyzed using a 4700 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems). Peptidic mass spectra were acquired in reflector positive mode at a 700–4000 m/z mass window, and proteins were identified by peptide mass fingerprint using Mascot software (Matrix Science, UK) integrated in the GPS Explorer software (ABSCIEX) and searched against the SwissProt/UniProt Homo sapiens protein sequence database. The maximum error tolerance was 35 ppm, and up to two missed cleavages were allowed.

Phosphopeptide Analysis by MS—Bands corresponding to NMHC-IIA, from anti-NMHC-IIA IPs of NI and L monocytogenes-infected HeLa cells, were processed as described above. Phosphopeptides were selected and enriched by titanium dioxide chromatography (TiO2 Mag-Sepharose, GE Healthcare). MALDI matrix used was 9 mg/ml 2',4',6'-trihydroxyacetophenone monohydrate, 5 mg/ml diammonium citrate, in 50:50, v/v, water/acetonitrile. Mass spectra were acquired in a 4800 Plus MALDI TOF/TOF analyzer mass spectrometer (AB SCIEX) both in reflector negative and MS/MS modes.

Immunoblotting—Proteins were resolved in SDS-polyacryl amide gels and transferred onto Nitrocellulose membranes (Hybond ECL, GE Healthcare). Membranes were blocked with 5% skimmed milk in buffer A (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, and 0.1% Triton X-100) for 1 h at room temperature or overnight at 4 °C. Primary and secondary antibodies were diluted in 2.5% skimmed milk in buffer A. Membranes used for anti-phosphotyrosine detection were blocked with Western Blocker solution (Sigma), also used to dilute primary and secondary antibodies.

Immunofluorescence Analysis—Cells were fixed in 3% paraformaldehyde (15 min), quenched with 20 mM NH₄Cl (1 h), permeabilized with 0.1% Triton X-100 (5 min), and blocked with 1% BSA in PBS (30 min). Antibodies were diluted in PBS containing 1% BSA. Coverslips were incubated for 1 h with primary antibodies washed three times in PBS and incubated 45 min with secondary antibodies and phalloidin Alexa 555 or 647. DNA was counterstained with DAPI (Sigma). Coverslips were mounted onto microscope slides with Aqua-Poly/Mount (18606, Polysciences). Images were collected with a confocal laser-scanning microscope (Zeiss Axiosvert LSM 510 or Leica SP2 AOBS S.E.) and processed using Adobe Photoshop software.

Kinase assay—Kinase assays were performed using the Src assay kit (17-131, Millipore), following the manufacturer’s instructions. Anti-GFP-immunoprecipitated fractions from HEK293 cells expressing GFP-NMHC-IIA-WT or NMHC-IIB depletion, using Lipofectamine RNAiMax (Invitrogen) following the manufacturer’s instructions. Assays were performed 48 h later. Sequences of siRNAs are provided in Table 4. For transient protein expression, HeLa cells were seeded in 24-well plates (1 × 10⁵ cells/well), 6-well plates (4 × 10⁵ cells/well), or 10-cm dishes (3 × 10⁶ cells/dish) and transfected at 90% confluence with 500 ng, 2.5 μg, or 15 μg of plasmid DNA, respectively, using Lipofectamine 2000 (Invitrogen). Assays were performed 24 h later. For rescue assays, HeLa cells were transfected with NMHC-IIA-si#2 and 24 h later transiently transfected with plasmids encoding siRNA-resistant GFP-NMHC-IIA-WT.
Src Kinase Phosphorylates NMHC-IIA upon Bacterial Infection

NMHC-IIA Is Tyrosine-phosphorylated in Response to Bacterial Infection—To identify new host proteins undergoing tyrosine phosphorylation (Tyr(P)) in response to L. monocytogenes and that could affect L. monocytogenes cellular infection, we compared the Tyr(P) protein profiles of L. monocytogenes-infected and noninfected (NI) HeLa cells. Cell extracts were collected at different time points post-inoculation and subjected to IP using anti-phosphotyrosine antibodies (anti-Tyr(P)). IP fractions were resolved by SDS-PAGE followed by silver staining. Bands showing variable intensities in L. monocytogenes-infected versus NI cells were excised and processed for mass spectrometry identification. A band corresponding to an ~250-kDa protein displaying increased intensity throughout the infection (Fig. 1A) was identified as the human NMHC-IIA (data not shown).

To validate this result, HeLa and Caco-2 cells were incubated with L. monocytogenes for different time periods, and the presence of NMHC-IIA in anti-Tyr(P) IP fractions was assessed by immunoblot using NMHC-IIA-specific antibodies. We detected a time-dependent increase of NMHC-IIA in IP fractions from L. monocytogenes-infected cells (Fig. 1B). Levels of NMHC-IIA in Tyr(P) fraction increased 3.5-fold after 60 min of L. monocytogenes incubation with HeLa cells and 15-fold in Caco-2 cells upon 20 min of L. monocytogenes infection (Fig. 1B). Levels of NMHC-IIA in whole cell lysates (WCL) were not affected by infection (Fig. 1B), showing that increased levels of NMHC-IIA in IP samples are not related to an augmentation of NMHC-IIA expression. Incubation of HeLa cells with the nonpathogenic species Listeria innocua for 60 min only induced a small enrichment of NMHC-IIA in the anti-Tyr(P) IP fractions as compared with L. monocytogenes (Fig. 1C). In addition, NMHC-IIA was barely detected in IP fractions from HeLa cells stimulated with E. coli DH5α or latex beads (Fig. 1C). Altogether, these results indicate that the enrichment of NMHC-IIA in the pool of Tyr(P) proteins is associated with the pathogenic features of L. monocytogenes and is not a broad cellular response to any extracellular stimuli.

To investigate whether the same response could be induced upon infection with other human bacterial pathogens, HeLa cells were incubated for 4 h with the extracellular pathogenic E. coli EPEC and EHEC or the invasive E. coli K12 expressing the Y. pseudotuberculosis invasin (K12-inv) (24), an infection model allowing the study of signaling pathways triggered downstream from the invasin-integrin interaction. As compared with NI conditions, NMHC-IIA appeared slightly increased in anti-Tyr(P) IP fractions from EPEC- and EHEC-infected cells. Strikingly, K12-inv induced a robust enrichment of NMHC-IIA in IP samples that is abolished in cells incubated with bacteria harboring a disrupted invasin-encoding gene

arbitrarily fixed to 100. Differences were not considered statistically significant for p value >0.05.

RESULTS

NMHC-IIA expression is tyrosine-phosphorylated in response to bacterial infection. To identify new host proteins undergoing tyrosine phosphorylation (Tyr(P)) in response to L. monocytogenes and that could affect L. monocytogenes cellular infection, we compared the Tyr(P) protein profiles of L. monocytogenes-infected and noninfected (NI) HeLa cells. Cell extracts were collected at different time points post-inoculation and subjected to IP using anti-phosphotyrosine antibodies (anti-Tyr(P)). IP fractions were resolved by SDS-PAGE followed by silver staining. Bands showing variable intensities in L. monocytogenes-infected versus NI cells were excised and processed for mass spectrometry identification. A band corresponding to an ~250-kDa protein displaying increased intensity throughout the infection (Fig. 1A) was identified as the human NMHC-IIA (data not shown).

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FIGURE 1. NMHC-IIA is tyrosine-phosphorylated in response to human bacterial pathogens. A, silver-stained acrylamide gel showing the tyrosine phosphorylation profiles of NI and L. monocytogenes-infected HeLa cells for the indicated periods of time. Total tyrosine-phosphorylated proteins were immunoprecipitated with an anti-Tyr(P) antibody. Molecular mass standards are indicated. Arrow shows a protein band with increased intensity over the time of infection and identified by mass spectrometry analysis as NMHC-IIA. B, HeLa and Caco-2 cells were left NI or incubated with L. monocytogenes and harvested at indicated time points post-infection (p.i.). Tyrosine-phosphorylated proteins were immunoprecipitated (IP: pTyr) from WCL, and NMHC-IIA was detected by immunoblot (NMHC-IIA) in IP fractions and WCL. Detection of actin protein levels served as loading control. Bottom panels show quantifications of NMHC-IIA signals from at least three independent experiments in WCL and IP fractions of NI and L. monocytogenes-infected HeLa (60 min p.i.) and Caco-2 (20 min p.i.) cells. C, HeLa cells were left NI or incubated with either L. monocytogenes (Lm), L. innocua (Li) (top panels), E. coli DH5α (Ec), or latex beads (bottom panels). Tyrosine-phosphorylated proteins were immunoprecipitated from WCL recovered at different time points and NMHC-IIA was analyzed by immunoblot (IB) in IP fractions and WCL. D, HeLa cells were left NI or incubated, for 4 h, with pathogenic E. coli (EPEC and EHEC) and E. coli K12 expressing a functional (inv) or mutated variant (Δinv) of Y. pseudotuberculosis invasin. Cells were also incubated with L. monocytogenes for 1 and 4 h (right panel). Tyrosine-phosphorylated proteins were immunoprecipitated and NMHC-IIA detected by immunoblot in IP fractions and WCL. Quantifications of NMHC-IIA signals for each IP fraction related to WCL are indicated (ratio). Values represent the mean of three independent experiments. E, NMHC-IIA was immunoprecipitated (IP: NMHC-IIA) from WCL of NI and L. monocytogenes-infected (Inf, 60 min) HeLa cells. Tyrosine-phosphorylated proteins (pTyr) and NMHC-IIA were detected in immunoprecipitates. As control, NMHC-IIA and actin were also detected in WCL.
Src Kinase Phosphorylates NMHC-IIA upon Bacterial Infection

**FIGURE 2. Activity of Src kinase is required for NMHC-IIA tyrosine phosphorylation upon *L. monocytogenes* cellular infection.** A, HeLa cells pretreated with PP1 (10 μM) or Y-27632 (50 μM) during 30 min were left NI or incubated with *L. monocytogenes* for 1 h (Inf) in the presence of the same concentrations of drugs. Nontreated (NT) HeLa cells were used as control. B, HeLa cells NT, transfected with a control empty plasmid (Ctr), or with an Src kinase-dead (Src-KD)-encoding plasmid. C, HeLa cells stably expressing an shRNA control (sh Ctrl) or a specific shRNA targeting Src expression (sh Src). Cells in B and C were left NI or incubated with *L. monocytogenes* for 1 h (Inf), 4–C, total tyrosine-phosphorylated proteins were immunoprecipitated, and NMHC-IIA was detected by immunoblot in IP fractions and WCL. Detection of actin levels served as loading control. Src protein levels were also confirmed by immunoblot. D, efficiency of Src depletion in sh Src HeLa cells was assessed by immunoblot using actin protein detection as loading control (left panel) and by quantitative RT-PCR (right panel). Src mRNA expression is represented relative to the expression of control HPRT1. In sh Ctrl cells, the relative expression was arbitrarily fixed to 1.

(K12-Δinv, Fig. 1D). For comparison, cells were also incubated with *L. monocytogenes* for 1 and 4 h (Fig. 1D). These results indicate that the enrichment of NMHC-IIA in the pool of Tyr(P) proteins is an event triggered by several human bacterial pathogens.

Our data suggest that bacterial infection either induces the direct NMHC-IIA Tyr(P) or stimulates its interaction with a protein that itself undergoes Tyr(P). To address this issue, endogenous NMHC-IIA was immunoprecipitated from NI and *L. monocytogenes*-infected HeLa cells, and Tyr(P) proteins were detected by immunoblot. A band showing a consistent 1.5-fold increase in intensity in infected samples was detected at the molecular weight of NMHC-IIA (Fig. 1E). Immunoprecipitated levels of NMHC-IIA were similar in NI and *L. monocytogenes*-infected cells. These results support a direct Tyr(P) of NMHC-IIA triggered by *L. monocytogenes* infection.

**NMHC-IIA-Tyr(P) Induced by *L. monocytogenes* Cellular Infection Requires the Activity of Src Tyrosine Kinase**—Considering our previous findings revealing the key role of the tyrosine kinase Src during *L. monocytogenes* invasion (7), we addressed the role of this kinase in NMHC-IIA-Tyr(P) in the context of *L. monocytogenes* infection. Prior to *L. monocytogenes* incubation, HeLa cells were treated with PP1, an inhibitor of Src family kinases, or with Y-27632, an inhibitor of the serine/threonine kinase ROCK that regulates NMHC-IIA activity through the phosphorylation of the regulatory light chain of myosin II and limits *L. monocytogenes* internalization (25). Given that NMHC-IIA-Tyr(P) is hardly detected by using anti-Tyr(P) antibodies in immunoblot, cell lysates were subjected to anti-Tyr(P) IP assay and NMHC-IIA was detected in IP fractions.

The increase in NMHC-IIA-Tyr(P) induced by *L. monocytogenes* infection of nontreated cells (NT) was abolished in PP1-treated cells while being not affected by Y-27632 treatment (Fig. 2A), suggesting that NMHC-IIA-Tyr(P) requires Src kinase activity and occurs independently from ROCK activity. In addition, we interfered with Src activity by overexpressing an Src kinase-dead variant (Src-KD) (26). Levels of NMHC-IIA-Tyr(P) induced by *L. monocytogenes* infection were assessed in HeLa cells nontransfected (NT), transfected, with an empty plasmid (Ctr), or with an Src kinase-dead (Src-KD) plasmid (Fig. 2A). Levels of NMHC-IIA-Tyr(P) induced by *L. monocytogenes* infection in cells overexpressing Src-KD the NMHC-IIA-Tyr(P) was almost undetectable (Fig. 2B). To further confirm these data, we targeted the expression of endogenous Src by using specific shRNAs. We observed that *L. monocytogenes*-induced NMHC-IIA-Tyr(P) occurred in shRNA control (sh Ctrl) and was clearly diminished in shRNA Src-expressing (sh Src) HeLa cells, in which Src expression is reduced by 60% (Fig. 2, C and D). Altogether, these data demonstrate that Src activity is required for NMHC-IIA-Tyr(P) triggered by bacterial infection.

**Host Src Kinase Phosphorylates NMHC-IIA in Tyrosine Residue 158**—The NMHC-IIA amino acid sequence includes 34 tyrosine residues, most of which are located in the myosin motor domain (Fig. 3A). To identify the NMHC-IIA tyrosine residues phosphorylated by Src upon *L. monocytogenes* infection, we used combined *in silico* approaches (NetPhos 2.0 and NetPhosK). Nine tyrosine residues were predicted as potentially phosphorylated, among which only the tyrosine in position 158 (Tyr-158) was a putative substrate for Src kinase (Fig.
To assess these in silico predictions and taking into account that \textit{L. monocytogenes}-induced NMHC-IIA-Tyr(P) requires Src kinase activity (Fig. 2), we determined whether NMHC-IIA-Tyr(P) occurs upon \textit{L. monocytogenes} infection of cells ectopically expressing either GFP-tagged NMHC-IIA-Y158F (in which Tyr-158 residue was replaced by a phenylalanine), NMHC-IIA-Y190F (harboring the same amino acid substitution in position 190, randomly selected, and unrelated to in silico predictions), or NMHC-IIA-WT (wild type NMHC-IIA). \textit{L. monocytogenes} infection of nontransfected (NT), NMHC-IIA-WT-, and NMHC-IIA-Y190F-overexpressing cells generated increased levels of NMHC-IIA-Tyr(P) as compared with NI cells, although the overexpression of NMHC-IIA-Y158F largely limited \textit{L. monocytogenes}-induced NMHC-IIA-Tyr(P) (Fig. 3C). Exogenous NMHC-IIA-WT and NMHC-IIA-Y190F were occasionally detected in anti-Tyr(P) IP fractions of \textit{L. monocytogenes} infection of NT, NMHC-IIA-WT-, and NMHC-IIA-Y190F-overexpressing cells generated increased levels of NMHC-IIA-Tyr(P) as compared with NI cells, although the overexpression of NMHC-IIA-Y158F largely limited \textit{L. monocytogenes}-induced NMHC-IIA-Tyr(P) (Fig. 3C). **FIGURE 3.** NMHC-IIA tyrosine residue in position 158 is phosphorylated in response to \textit{L. monocytogenes} infection. A, schematic representation of NMHC-IIA showing the distribution of tyrosine residues (red bars). Tyrosine residues in position 158 (Y158) and 190 (Y190) are highlighted. ATP-binding site, motor, and tail domains are indicated. B, in silico predictions obtained from NetPhos 2.0 and NetPhosK servers, for tyrosine phosphorylation potential (score) and putative kinase involved. The position and amino acid environment of tyrosine residues showing a phosphorylation potential above the threshold (score >0.5) are indicated. C, HeLa cells expressing the wild type GFP-NMHC-IIA-WT (WT) and the mutants GFP-NMHC-IIA-Y158F (Y158F) or GFP-NMHC-IIA-Y190F (Y190F) were left NI or incubated with \textit{L. monocytogenes} for 1 h (Inf). NMHC-IIA was detected by immunoblot in anti-Tyr(P) immunoprecipitates and WCL. Detection of GFP indicates similar expression levels of NMHC-IIA constructs and actin levels served as loading control. D, HeLa cells were left NI or incubated with \textit{L. monocytogenes} for 1 h (Inf). Total cell extracts were used in immunoblot using an antibody raised against NMHC-IIA-Tyr(P)-158. Total levels of NMHC-IIA were detected using an anti-NMHC-IIA antibody, and \(\alpha\)-tubulin levels were used as loading control. Bottom panel shows quantification of NMHC-IIA-Tyr(P)-158 signals from three independent experiments in NI and \textit{L. monocytogenes}-infected HeLa cells. Two peak clusters marked as I (monoisotopic peak at m/z 3025.37 [M – H]\(^{-}\)) and II (monoisotopic peak at m/z 3041.36 [M – H]\(^{-}\) with oxidized methionine) were detected. The corresponding NMHC-IIA peptide (amino acid 142–165) is indicated, and Tyr-158 is highlighted. The area of the clusters in NI and infected conditions is indicated in parentheses. F, anti-GFP IP fractions obtained from WCL of HEK293 cells expressing either GFP-NMHC-IIA-WT (WT) or GFP-NMHC-IIA-Y158F (Y158F) were used in vitro Src kinase assays. A synthetic peptide was used as positive control (Ctr). Incorporation of radiolabeled \(\gamma\)\(^{32}\)P]ATP was measured in counts/min for each condition. Results are representative of two independent experiments. G, comparative analysis of the NMHC-IIA amino acid sequence from different species, focused in the region encompassing the tyrosine on position 158.
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**FIGURE 4.** *L. monocytogenes* intracellular levels increased upon inhibition of NMHC-IIA activity. A, levels of intracellular *L. monocytogenes* (Lm) were assessed by gentamicin protection assay and CFU counting in HeLa and Caco-2 cells treated with 10 or 100 μM blebbistatin (Blebb). Graph shows the fold increase of intracellular *L. monocytogenes* determined as the ratio of intracellular bacteria in cells treated with the active versus the inactive enantiomer of blebbistatin. B, single confocal sections of HeLa cells infected with *L. monocytogenes* in the presence of DMSO (control) or 50 μM active blebbistatin. Infected cells were immunolabeled for NMHC-IIA (green) and *L. monocytogenes* (blue) and stained for actin (red). C, immunofluorescence scoring of DMSO- and active blebbistatin-treated HeLa cells associated with *L. monocytogenes* and showing *L. monocytogenes*-associated actin foci. Results are means ± S.D. from three independent experiments, each done in duplicate. Statistically significant differences are indicated: *, p < 0.05.

Monocytogenes-infected cells (data not shown). Levels of endogenous NMHC-IIA were comparable in the different conditions, and GFP fusion proteins were expressed similarly in NI and infected cells (Fig. 3C). These results corroborate in silico predictions and suggest the central role of Tyr-158 in NMHC-IIA-Tyr(P) triggered upon infection. To validate our results, total lysates from NI and *L. monocytogenes*-infected cells were probed with an antibody raised against a peptide comprising the phosphorylated Tyr-158 residue of NMHC-IIA (Tyr(P)-158). In agreement with our data, levels of NMHC-IIA-Tyr(P)-158 were increased 4.8-fold in *L. monocytogenes*-infected cells (Fig. 3D). In addition, samples enriched in NMHC-IIA phosphopeptides from NI and *L. monocytogenes*-infected cells were analyzed by mass spectrometry. A phosphopeptide spanning Tyr-158 (amino acids 142–165, KRHEMPHPHYAIDT-TAYRSMMQDR) was detected at m/z 3025.37 [M – H]⁻ (Fig. 3E, cluster I) and at 3041.36 [M – H]⁻ with an oxidized methionine (Fig. 3E, cluster II). In infected samples, the area of cluster I that is correlated with the abundance of the corresponding phosphopeptide was increased 4.8-fold. Cluster II appeared 2.1-fold more abundant in *L. monocytogenes*-infected samples as compared with NI. Cluster I was further characterized and validated by MS/MS sequencing. Altogether, our data show that phosphorylation occurs at Tyr-158.

We further evaluated whether NMHC-IIA-Tyr(P) occurs specifically on Tyr-158 by culturing *L. monocytogenes* WT or Y158F with GFP fusion proteins of Src kinase, the control peptide (Ctr) and IP fractions of NMHC-IIA-WT and Y158F showed residual levels of [γ-32P]ATP incorporation. In the presence of Src kinase, the NMHC-IIA-WT-enriched IP fraction and the control peptide became radiolabeled, whereas the radioactivity incorporation in the NMHC-IIA-Y158F enriched sample remained at a basal level (Fig. 3F).

Altogether these results strongly suggest that Tyr-158 of NMHC-IIA is a substrate for Src kinase, becoming phosphorylated in response to *L. monocytogenes* infection, and put forward the putative role of this event in cellular infection. In addition, Tyr-158 appears extremely conserved among species ranging from *Saccharomyces cerevisiae* to *H. sapiens* (Fig. 3G), pointing to the broad importance for Tyr-158 in the regulation of highly conserved canonical functions of NMHC-IIA.

**Inhibition of NMHC-IIA Activity Affects Intracellular Levels of L. monocytogenes**—To assess the role of NMHC-IIA activity in cellular infection, we measured intracellular levels of *L. monocytogenes* following chemical inhibition of NMHC-IIA. Blebbistatin, a specific inhibitor of myosin II activity (27), was added (10 or 100 μM) to HeLa and Caco-2 cells, and *L. monocytogenes* infection efficiency was quantified by gentamicin protection assays. As control, we used an inactive form of blebbistatin. *L. monocytogenes* intracellular levels were increased by 2–8-fold, in a dose-dependent manner in both cell lines, following treatment with the active as compared with the inactive enantiomer of blebbistatin (Fig. 4A). Untreated and inactive blebbistatin-treated cells showed similar levels of intracellular *L. monocytogenes* (data not shown). Our data are in agreement with a previous report showing that blebbistatin treatment of L2 cells increases *L. monocytogenes* adhesion and invasion (25). Recruitment of NMHC-IIA and formation of actin foci at *L. monocytogenes* entry sites were both detected in control (DMSO) and active blebbistatin-treated HeLa cells (Fig. 4B). Although the percentage of *L. monocytogenes*-associated cells remained similar in both conditions, the percentage of cells showing *L. monocytogenes*-actin foci increased in the presence of active blebbistatin (Fig. 4C). Together, our results indicate that the ATPase activity of NMHC-IIA is not required for its localization to the sites of *L. monocytogenes* uptake and does not influence the interaction of *L. monocytogenes* with host cells. However, inhibition of NMHC-IIA ATPase activity fosters the formation of *L. monocytogenes*-actin foci, which correlates with increased rates of intracellular bacteria.

**Reduced Expression of NMHC-IIA Increases the Level of Intracellular L. monocytogenes**—To further address the role of NMHC-IIA in *L. monocytogenes* cellular infection, levels of adherent and intracellular *L. monocytogenes* were quantified by gentamicin protection assays in NMHC-IIA-depleted HeLa cells, using two siRNAs (si#1 and si#2). In accordance with the data described above, levels of intracellular *L. monocytogenes* increased 2-fold in NMHC-IIA-depleted (IIA-si#1 and IIA-si#2) as compared with control siRNA-transfected cells (Ctr).
NMHC-IIA depletion assessed by immunoblot reached 85% in si#1-transfected cells and 65% when using si#2 (Fig. 5A). Levels of adhered *L. monocytogenes* were also augmented in NMHC-IIA-depleted cells (data not shown). Immunofluorescence analysis of *L. monocytogenes*-infected NMHC-IIA-depleted cells revealed a 2-fold increase in the percentage of cells showing *L. monocytogenes*-associated actin foci, evaluated by immunofluorescence scoring. 

**FIGURE 5. Depletion of NMHC-IIA facilitated *L. monocytogenes* cellular infection.** A, intracellular levels of *L. monocytogenes* (*Lm*) assessed by gentamicin protection assay in HeLa cells NT or transfected with either control siRNA (Ctr) or NMHC-IIA-specific siRNAs (si#1 and si#2). Efficiency of NMHC-IIA knockdown was assessed by immunoblot and quantified. Indicated values (normalized expression) are relative to actin and NMHC-IIA expression levels in NT cells. B, percentage of control (Ctr) or NMHC-IIA-depleted cells (IIA-si#1) associated with *L. monocytogenes* and showing *L. monocytogenes*-associated actin foci, evaluated by immunofluorescence scoring. C, number of bacteria and actin foci per cell in control and NMHC-IIA-depleted conditions. D, depletion of NMHC-IIA does not affect the expression of NMHC-IIB. NMHC-IIB expression levels were evaluated by immunoblot in NMHC-IIA-depleted (IIA-si#1) as compared with control (NT and Ctr) cells. Actin was used as loading control. E, intracellular levels of *L. monocytogenes* were assessed by gentamicin protection assay in HeLa cells transfected with either control siRNA (Ctr) or NMHC-IIB-specific siRNA (IIB-si). Efficiency of NMHC-IIB knockdown was assessed by immunoblot using actin protein detection as loading control. F, expression of NMHC-IIA was restored in si#2-depleted cells through the expression of a siRNA-resistant GFP-NMHC-IIA (NMHC-IIA-siRes). Intracellular levels of *L. monocytogenes* assessed by gentamicin protection assay in HeLa cells expressing different levels of NMHC-IIA are shown. Nontreated and NMHC-IIA-depleted cells expressing a wild type GFP-NMHC-IIA (NMHC-IIA-WT) were used as controls. Endogenous NMHC-IIA silencing and GFP-NMHC-IIA expression was evaluated by immunoblot. Detection of actin levels served as loading control. G, Western blot showing expression levels of endogenous (anti-NMHC-IIA, M8064, Sigma) and ectopically expressed NMHC-IIA (anti-GFP, B2, Santa Cruz Biotechnology) in HeLa cells transfected either with GFP-NMHC-IIA-WT or GFP-NMHC-IIA-siRes expression vectors. H, intracellular levels of *L. innocua* expressing inlB (*L. innocua* (Li)-inlB), *E. coli* K12 expressing the invasin (K12-inv), and *Y. pseudotuberculosis* (Yp) were assessed by gentamicin protection assay in HeLa cells transfected with either control siRNA (Ctr) or NMHC-IIA-specific siRNA (IIA-si#1). A and F, number of intracellular *L. monocytogenes* in NT cells was normalized to 100%, and those in siRNA-transfected cells were expressed as relative values to NT cells. E and H, numbers of intracellular bacteria were normalized to 100% in Ctr cells and expressed as relative values in the other conditions. Results shown in A–C, E, F, and H are means ± S.E. of at least three independent experiments, each done in triplicate. Statistically significant differences are indicated: *, *p < 0.05; **, *p < 0.01 and ***, *p < 0.001.
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FIGURE 6. NMHC-IIA phosphorylation in tyrosine 158 is required to limit L. monocytogenes cellular infection. Intracellular levels of L. monocytogenes were assessed by gentamicin protection assays in the presence of 10 μM PP1 (A) or in HeLa cells expressing Src-KD (B). C, levels of intracellular K12-inv were assessed by gentamicin protection assay in HeLa cells treated with 10 μM of PP1. D, intracellular levels of L. monocytogenes were assessed by gentamicin protection assays in HeLa and COS-7 cells expressing either GFP-NMHC-IIA-WT (WT) or GFP-NMHC-IIA-Y158F (Y158F). Results shown in A–D are means ± S.E. of three independent experiments, each done in triplicate. Numbers of intracellular bacteria were normalized to 100% in control cells and expressed as relative values.

of cells associated with L. monocytogenes and a 3-fold increase in the percentage of cells showing L. monocytogenes-associated actin foci (Fig. 5B). The number of bacteria and actin foci per cell were also increased in NMHC-IIA-depleted cells (Fig. 5C), correlating with increased levels of intracellular bacteria. Our data indicate that, although L. monocytogenes association with cells does not require NMHC-II activity, it is modulated by NMHC-II itself probably through the interaction with other proteins.

To discard the hypothesis that increased levels of intracellular L. monocytogenes detected in NMHC-IIA-depleted cells could result from the overexpression of the isoform B of non-muscle myosin heavy chain (NMHC-IIIB), we confirmed that expression levels of NMHC-IIIB were similar in NMHC-IIA-depleted and control cells (Fig. 5D). However, in the absence of si#2, both NMHC-IIA-WT and siRes variants are expressed at similar levels (Fig. 5G). Together, these results confirm that the increase in L. monocytogenes intracellular levels observed in NMHC-IIA-depleted cells is specifically due to NMHC-IIA depletion.

To analyze whether the role of NMHC-IIA on intracellular levels of bacteria was specific for L. monocytogenes or could be broadened to other bacterial infectious processes, we performed gentamicin protection assays using L. innocua expressing InlB (L. innocua-inlB), the major internalin driving L. monocytogenes entry in HeLa cells (28), K12-inv, and Y. pseudotuberculosis. Numbers of intracellular L. innocua-inlB were not significantly different in NMHC-IIA-depleted and Ctr cells (Fig. 5H). In contrast, levels of intracellular K12-inv and Y. pseudotuberculosis were significantly lower in NMHC-IIA-depleted cells (Fig. 5H). Our data indicate that NMHC-IIA is specifically triggered by pathogenic L. monocytogenes and is independent of an InlB-mediated uptake. In contrast, the invasin-mediated uptake requires NMHC-IIA. Interestingly, NMHC-IIA and -IIB were shown to be required for SopB-mediated invasion of Salmonella (18). Our findings, together with published reports, reveal that NMHC-IIA plays opposite roles in different infection models; although it is required for an utmost Y. pseudotuberculosis and Salmonella infection, it has a restrictive role in L. monocytogenes cellular infection.

Function of NMHC-IIA in L. monocytogenes Infection Relies on the Phosphorylation of Its Tyrosine 158 —We reported above two important observations. 1) NMHC-IIA is tyrosine-phosphorylated by Src kinase upon L. monocytogenes incubation with cells. 2) L. monocytogenes intracellular levels are increased in conditions of NMHC-IIA depletion or inhibition of its activity, demonstrating that NMHC-IIA activity limits L. monocytogenes infection. To investigate whether both findings could be interconnected, we evaluated levels of intracellular bacteria...
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under conditions where NMHC-IIA-Tyr(P) does not occur. We used cells with compromised Src activity (PP1 treatment and Src-KD overexpression) and cells expressing an NMHC-IIA nonphosphorylatable variant (NMHC-IIA-Y158F). Levels of intracellular *L. monocytogenes* showed a 2.5-fold increase in PP1-treated HeLa cells as compared with control DMSO-treated cells (Fig. 6A). In agreement, we observed an increase in *L. monocytogenes* intracellular levels in cells expressing Src-KD (Fig. 6B). Inversely, intracellular levels of K12-inv decreased 2-fold in PP1-treated cells (Fig. 6C), as reported previously (29).

Increased levels of intracellular *L. monocytogenes* detected in conditions of Src inactivation and thus in the absence of NMHC-IIA-Tyr(P) correlate with our data showing that reduced levels or inactivation of NMHC-IIA resulted in increased numbers of intracellular *L. monocytogenes*. Our data also suggest an association between the role of NMHC-IIA in *Y. pseudotuberculosis* invasin-mediated uptake and invasin-triggered NMHC-IIA-Tyr(P).

To further confirm the role of NMHC-IIA-Tyr(P) in the *L. monocytogenes* cellular infection, we evaluated intracellular levels of *L. monocytogenes* in HeLa and COS-7 cells transiently expressing either the GFP-NMHC-IIA-WT (WT) or the nonphosphorylatable variant GFP-NMHC-IIA-Y158F (Y158F). In contrast to HeLa cells, COS-7 cells naturally lack NMHC-IIA expression, thus appearing as a valuable experimental model to contrast to HeLa cells, COS-7 cells naturally lack NMHC-IIA phosphorylatable variant GFP-NMHC-IIA-Y158F (Y158F). In increased numbers of intracellular *L. monocytogenes*. Our data also suggest an association between the role of NMHC-IIA in *Y. pseudotuberculosis* invasin-mediated uptake and invasin-triggered NMHC-IIA-Tyr(P).

DISCUSSION

Pathogens interfere with host phosphorylation cascades to foster adhesion, invasion, and intracellular survival. Here, we searched for new host proteins undergoing tyrosine phosphorylation upon *L. monocytogenes* infection. We showed that NMHC-IIA is tyrosine-phosphorylated in response to *L. monocytogenes* as well as to other human bacterial pathogens such as EPEC, EHEC, and K12-inv. In *L. monocytogenes* infection, this previously unknown tyrosine phosphorylation event is triggered by Src kinase on residue Tyr-158 of NMHC-IIA, and it limits intracellular bacterial levels.

Myosin II activity is regulated by phosphorylation events in serine and threonine residues of the regulatory light chain (15). NMHC-IIA also undergoes serine and threonine phosphorylations, which regulate the assembly of myosin II filaments in vitro and are thought to control subcellular localization of NMHC-IIA and contractility that depends on the actin cross-linking activity of NMHC-IIA (15). Although NMHC-IIA was detected in studies aiming to unravel the global phosphorylation signaling in cancer tissues (30, 31), its tyrosine phosphorylation has never been characterized. Our data constitute the first report showing and characterizing NMHC-IIA-Tyr(P).

Our preliminary in silico analysis suggests an important and broad role for NMHC-IIA Tyr(P) in position 158 as follows. 1) Tyr-158 is highly conserved among species ranging from *S. cerevisiae* to *H. sapiens*. 2) An in silico study suggested that Tyr-163 of muscle myosin heavy chain (matching Tyr-158 in NMHC-IIA) could be phosphorylated (32). 3) Tyr-158 is located in the motor domain of NMHC-IIA near the ATP-binding pocket. 4) Analysis of the crystal structure of the myosin motor domain (33) showed that Tyr-158 is exposed at the surface of the protein and is thus accessible for phosphorylation. Thus, we hypothesize that the phosphorylation of NMHC-IIA Tyr-158 could modulate NMHC-IIA activity most probably by affecting its ability to bind and/or hydrolyze ATP. However at this point any other mechanism could be envisaged. In addition, it is likely that NMHC-IIA-Tyr(P) in Tyr-158 occurs in specific physiological conditions engaging NMHC-IIA activity and thus plays a role in the regulation of the highly conserved canonical functions of NMHC-IIA. The functional and structural outcomes of such modification are now critical to elucidate.

Our data suggest that, upon infection, only a small pool of NMHC-IIA becomes phosphorylated in Tyr-158, probably concentrated in a restricted subcellular localization and/or interacting with specific partners, which would impact infection. Yet, we observed that both NMHC-IIA-WT and Y158F concentrated around bacteria at the entry site. We also found that phosphorylation of Tyr-158 does not affect the phosphorylation of the myosin regulatory light chain,7 which is achieved by MLCK and is required for activation of myosin II motor activity (15). Interestingly, Src was previously shown recruited to membrane blebs where it associates with MLCK and myosin II (34, 35). In response to cell swelling, Src and MLCK form a complex in which Src activates MLCK, and both regulate a compensatory membrane retrieval that requires myosin II (35). It is thus conceivable that Src and MLCK could work together to fine-tune the activity of myosin II in the context of infection.

Myosin II isoforms were recently involved in viral and bacterial infections either promoting or limiting pathogen progression. However, their role in such processes is still mainly descriptive. NMHC-IIA is required for Kaposi sarcoma-associated herpesvirus and HSV1 entry into cells (16, 17, 36), facilitates *Salmonella* invasion, and regulates its intracellular growth (18, 37) and promotes *Chlamydia* dissemination (19). Conversely, myosin II limits bacterial cell-to-cell spread by restraining *L. monocytogenes* protrusion formation (38) and participating in the formation of *Shigella*-associated septin cages (39). NMHC-IIIB is involved in the formation of actin-rich structures that accumulate near the *Salmonella*-containing vacuole and

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restrain bacterial intracellular multiplication (40). Altogether, these data suggest that the different outcomes associated with myosin II function during infection are probably related to the cellular machinery engaged in the various infectious processes. Our results indicate that NMHC-IIA activity limits L. monocytogenes infection most probably hindering cellular invasion by interfering with the formation of L. monocytogenes-induced actin foci. NMHC-IIA-depleted or inactivated cells were reported to lose cytoplasm cohesion and show increased membrane activity and plasticity (41, 42). These phenotypes could thus suggest that the increased numbers of intracellular L. monocytogenes observed in such cells would be greatly due to the disruption membrane rigidity. However, if this was the case, cells displaying low NMHC-IIA activity should be more permissive to any extracellular pathogen, which was not observed in KSHV (17), HSV1 (16), and Salmonella (18) infections. In addition, we show here that NMHC-IIA sustains invasin-mediated Y. pseudotuberculosis infection, and the invasion rate of L. innocua expressing InlB was not significantly increased by NMHC-IIA depletion, thus excluding a nonspecific cell invasion mechanism.

NMHC-IIA participates in cellular processes associated with phosphorysine signaling, which are largely usurped by bacteria, namely L. monocytogenes and Y. pseudotuberculosis (43), during infection. NMHC-IIA regulates protrusion formation and cell migration through the generation of actin retrograde flow (44, 45); it is required for integrin-mediated adhesion maturation (46); it controls cell-cell adhesion promoting E-cadherin clustering and stabilizing cellular junctions (47); and it governs the polarization of epithelial cells generating forces to maintain the epithelia (48). Whether NMHC-IIA is Tyr(P) in these processes is unknown.

In intercellular junctions, NMHC-IIA is critical for the E-cadherin localization (47), and Src activation is required for actin polymerization at cell-cell contacts (49) as it is during E-cadherin-mediated L. monocytogenes invasion (7). Interestingly, Src activation and recruitment of c-Cbl are key events to control c-Met signaling (50). Our data show that Src activity restricts intracellular levels of L. monocytogenes in HeLa cells in which L. monocytogenes uptake is mainly mediated by c-Met and present the hypothesis that Src is acting through the tyrosine phosphorylation of NMHC-IIA to inhibit entry. Remarkably, in KSHV infection, which depends on integrin and Src activation (51), NMHC-IIA interacts with the ubiquitin ligase c-Cbl (17). The complex c-Cbl-NMHC-IIA associates with the receptor tyrosine kinase EphA2 that amplifies Src signaling to promote viral macropinocytosis (36). It is thus possible that c-Cbl, which is required for L. monocytogenes infection (52), associates with NMHC-IIA and c-Met to modulate L. monocytogenes infection through tyrosine phosphorylation events. To invade cells, Y. pseudotuberculosis binds β1-integrin (53), which interacts with NMHC-IIA via its cytoplasmic tail to regulate cell migration (54). As in adhesion and cell migration processes (55), during Y. pseudotuberculosis infection the engagement of β1-integrin leads to the activation of Src kinase (56), which could also act on NMHC-IIA triggering its tyrosine phosphorylation at the site of bacterial attachment thereby promoting Y. pseudotuberculosis infection.

Our data open new perspectives in the regulatory mechanisms governing NMHC-IIA functions in infection and physiological cellular processes. Further work should reveal whether NMHC-IIA-Tyr(P) affects its motor activity, binding partners, and/or the formation of actomyosin filaments.

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