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Short Communication

HopQ impacts the integrin α5β1-independent NF-κB activation by *Helicobacter pylori* in CEACAM expressing cells

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ABSTRACT

Helicobacter pylori infection persists in more than half of the world's population and represents a risk factor for peptic ulcer disease and gastric cancer. Virulent strains of *H. pylori* carry a *cag* pathogenicity island (*cag*PAI), which encodes a type IV secretion system (T4SS) with the capability to inject the effector protein cytotoxin-associated gene A (CagA) into eukaryotic cells. Colonisation of the gastric epithelium by *H. pylori* provokes direct activation of the proinflammatory and survival factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). We investigated the impact of host cell receptor integrin α5β1 and the bacterial adhesin HopQ on the NF-κB activation. We found that *H. pylori* induced early T4SS-dependent, but CagA-independent canonical NF-κB signalling in polarized, apical infected NCI-N87 cells. Integrin-dependent CagA translocation was hardly detectable, as integrin β1 was sparsely located at the apical surface of polarized NCI-N87 cells. Knockdown experiments indicated that integrin α5β1 and integrin linked kinase (ILK) were dispensable for NF-κB activation in *H. pylori* infection. Thus, there exists no common mechanism, which mediates integrin α5β1-dependent *H. pylori*-triggered CagA translocation and the activation of NF-κB. Further, we report that *H. pylori* adhesin HopQ, which binds to a specific subset of carcinoembryonic antigen-related cell adhesion molecules (CEACAMs), promotes canonical NF-κB activation in AGS and NCI-N87 cells, but not in HeLa cells, which are devoid of these CEACAMs. Noteworthy, these effects were not mediated by reduced adhesion, indicating additional functions of HopQ.

1. Introduction

Over fifty percent of the world population is infected with *H. pylori*, one of the etiological risk factors in gastritis, gastric ulcer and gastric adenocarcinoma (Plummer et al., 2015). *H. pylori* strains, which possess the *cag*PAI encode a T4SS to inject the effector protein CagA into the cytosol of eukaryotic cells, where it becomes tyrosine phosphorylated by Src family kinases (Naumann et al., 2017).

A postulated host cell receptor for T4SS-dependent CagA translocation is integrin α5β1 (Kwok et al., 2007), but integrin β1 overexpression in cell lines devoid of it, is sufficient to mediate CagA translocation (Jimenez-Soto et al., 2009). Integrins are transmembrane receptors that consist of α and β subunits, of which 24 different αβ heterodimers are known in mammals (Hynes, 2002). The cytoplasmic domain of integrin β1 interacts with the integrin linked kinase (ILK), which can trigger outside-in signalling involved in cell-matrix interactions and cytoskeletal organization (Cabodi et al., 2010). Interaction studies have shown, that the T4SS surface components CagI and CagY have the capacity to interact with integrin β1 heterodimers, however the putative interactor CagL has been studied in most detail (Kwok et al., 2007; Jimenez-Soto et al., 2009). CagL supposedly interacts in an arginine-glycine-aspartate (RGD) motif-dependent manner with integrins (Kwok et al., 2007; Bonsor et al., 2015), where the two variable CagL motifs TSPSA and TASLI, opposite of the RGD motif, can be crucial for the interaction (Bonig et al., 2016). It is mentionable, that CagL has the capability to interact *in vitro* with diverse integrins with high affinity, *e.g.* with integrin αVβ6 (Barden and Niemann, 2015). However, the functional relevance of these interactions for CagA translocation remains to be determined.

Besides the T4SS, *H. pylori* encodes numerous outer membrane proteins (OMPs). These proteins belong mostly to two families: the *Helicobacter* outer membrane protein (Hop) family and the Hop-related proteins (Hor). The Hop family encompasses adhesins like blood group antigen binding adhesion (BabA), sialic acid binding adhesin (SabA),

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adherence-associated lipoproteins A and B (AlpA and AlpB) and HopQ (da Costa et al., 2015). It has been proposed, that HopQ mediates, among others, the adherence of *H. pylori* to gastric epithelial cells by binding to the CEACAM receptors 1, 3, 5 and 6. Consistently, deletion of HopQ reduces CagA translocation to the host cell cytoplasm (Königer et al., 2016; Javaheri et al., 2016).

Infection of gastric epithelial cells with *H. pylori* induces secretion of proinflammatory cytokines, *e.g.* interleukin-8 (IL-8), which is transcriptionally regulated by NF-κB (Naumann et al., 2017). The NF-κB family consists of RelA, RelB, c-Rel, NF-κB1/p50 and NF-κB2/p52 proteins. The transcription factors are present in the cytoplasm and repressed by inhibitors of NF-κB (IκBs). Activation of canonical NF-κB by certain stimuli is rapid, and involves phosphorylation and degradation of IκBα. The released NF-κB heterodimer composed of RelA and p50 enters the nucleus and regulates expression of target genes (Neumann and Naumann, 2007). Using AGS cells, it has been shown that *H. pylori*-mediated activation of canonical NF-κB and IL-8 expression is T4SS-dependent, but CagA independent at early infection time points (Schweitzer et al., 2010; Sokolova et al., 2013).

Our data show, that in both non-polarized and polarized NCI-N87 cells, the fast canonical NF-κB activation depends on the T4SS, but is independent of CagA. We provide evidence that the NF-κB activation does not require integrin α5β1 and integrin downstream kinase ILK. Further, HopQ promotes canonical NF-κB activation in NCI-N87 and AGS, but not in HeLa cells, which could be correlated with CEACAM1, 5 and 6 expression in NCI-N87 and AGS cells.

2. Materials and methods

2.1. Cell culture and bacteria

NCI-N87 (ATCC, CRL-5822), AGS (ATCC, CRL-1739) and HeLa (ATCC, CCL-2) cell lines were maintained in RPMI 1640 medium (gibco®/Life Technologies) supplemented with 10% fetal calf serum (FCS) at 37 °C in a humidified 5% CO_2 atmosphere. The cell medium was replaced with RPMI 1640 supplemented with 0.2% FCS 14–17 h prior infection. To obtain a confluent polarized cell monolayer we used NCI-N87 cells as described by Diesing et al. (2013). 2×10^5 cells were seeded on porous ThinCert™ 12-well inserts (culture surface 1.13 cm^2, pore size 1 µm, Greiner Bio-One) and cultivated for 6 days. The monolayer formation was monitored by measuring the transepithelial electrical resistance (TER) using the Millicell®- ERS (Millipore).

H. pylori strains P1 (wt, *cagA*, *virB7*) (Backert et al., 2000), P12 (wt, *hopQ*, *hopQ*::*hopQ*I, *virB7*) (Königer et al., 2016) and *H. pylori* P1 wt variant, which expresses the green fluorescent protein (GFP) (Backert et al., 2005), were grown on GC agar plates supplemented with 10% horse serum, 5 µg/ml trimethoprim, 1 µg/ml nystatin, 10 µg/ml vancomycin under microaerophilic conditions at 37 °C for 48 h. The *cagA, virB7* and *hopQ* mutants as well as the GFP-expressing P1 strain were grown in presence of 6 µg/ml chloramphenicol in addition. For growing of the P12 *hopQ*::*hopQ*I, 8 µg/ml kanamycin was added to the plates. Bacteria were harvested in phosphate-buffered saline (PBS) and eukaryotic cells were infected at a multiplicity of infection (MOI) of 100 (if not stated otherwise). Prior the harvest, cultured cells were washed twice with PBS to remove unattached bacteria. To determine the number of cell-adherent *H. pylori*, infected NCI-N87 cells were washed twice with the RPMI 1640 at 3 h post infection, incubated in 1 ml of RPMI 1640 supplemented with 0.05% saponin for 5 min and collected. The samples were serially diluted with pre-warmed PBS, and the appropriate dilutions were plated on GC agar plates in duplicates. One week later, *H. pylori* colony-forming units (CFU) were counted.

2.2. siRNA transfection

At 24 h post-seeding of the cells, the cell culture medium was changed to Opti-MEM (gibco®/Life Technologies). 40 nM of siRNA targeting integrin α5 (pool of 4 siRNAs, Dharmacon™/GE Healthcare, L-008003-00), integrin β1 (pool of 4 siRNAs, Dharmacon™/GE Healthcare, L-004506-00) or 70 nM of siRNA targeting ILK (Santa Cruz Biotechnology, sc-35666) were mixed with 10 µl of siLentFect™ transfection reagent (BioRad) in 200 µl Opti-MEM, and added to the cells. After 2 h, FCS was added to the cells to achieve a final concentration of 10%. The incubation with siRNA complexes took place for 48 h for the knockdown of integrins and 72 h for the knockdown of ILK. Scrambled siRNA (Qiagen, 1027280) was used as control.

2.3. Immunofluorescence (IF)

For IF experiments, cells grown on membranes of ThinCert™ cell culture inserts were infected with GFP-expressing *H. pylori* P1 wt. The cells were fixed with 4% PFA for 15 min and afterwards washed thrice with PBS. The ThinCert™ membranes with fixed cells on their surface were carefully detached from the housing and blocked in TBS buffer containing 10% FCS and 0.25% Triton X-100 for 1 h at room temperature. Subsequently, specimens were incubated with the indicated primary antibody in TBS buffer containing 10% FCS and 0.25% TritonX-100 blocking solution over night at 4 °C, washed thrice with PBS and afterwards incubated with the secondary antibody in TBS-Tween buffer containing 1% BSA for 1.5 h at room temperature. After washing thrice with TBS-Tween, propidium iodide or Syto59 were added to stain the nuclei. The following primary antibodies were used: ezrin (Santa Cruz Biotechnology, sc-58758) and integrin β1 (Abcam, ab21845). Images were taken with Zeiss Axiovert 100 M confocal laser scanning microscope LSM 510 and processed with LSM image browser software.

2.4. Preparation of cell lysates, immunoblotting and immunoprecipitation

For immunoblot analysis whole cell lysates were prepared with a modified RIPA buffer (Sokolova et al., 2008). Samples were boiled with Laemmli buffer for 5 min and separated by SDS-PAGE, transferred onto PVDF membranes (Millipore) and blocked for 1 h at room temperature with 5% non-fat milk in TBS-Tween buffer. The membranes were incubated with indicated primary antibody in TBS-Tween containing either 5% non-fat milk or 5% BSA overnight at 4 °C and subsequently with an appropriate HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories). Immunoblots were developed using a chemiluminescence substrate (Millipore). The following primary antibodies were used: RelA (BD Biosciences Pharmingen, 610869), GAPDH (Millipore, MAB374), CagA (Austral Biologicals, HPM-5001-5), flagellin (Acris Antibodies GmbH, AM00865PU-N). p-RelA (3031), p-Tyr-100 (9411), p-IκBα (9246), IκBα (4812) and ILK (3856) antibodies were from Cell Signaling Technology. Integrin α5 (sc-10729) and integrin β1 (sc-6622) antibodies were from Santa Cruz Biotechnology.

For immunoprecipitation, whole cell lysates were incubated with CagA antibody (0.2 µg antibody/100 µg protein) overnight under rotation at 4 °C. Pre-washed protein G Sepharose 4 Fast Flow beads (50 µl of a 50% suspension, GE Healthcare) were added to the lysate to capture the immunocomplex. The beads were washed four times using RIPA buffer and the immunocomplex was eluted in 2× Laemmli sample buffer for further analysis.

2.5. Densitometric analysis

The densitometric quantification of band intensities was performed using the ImageJ software according to Schneider et al. (2012).

2.6. PCR amplification of CEACAM1, 3, 5, 6

The total RNA from AGS, NCI-N87, MKN45 and HeLa cells was isolated using the NucleoSpin® RNAII kit (Macherey-Nagel, Germany). Total RNA from leukocytes, prepared from whole blood, was used for