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Non-T cell activation linker regulates ERK activation in *Helicobacter pylori*-infected epithelial cells

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ABSTRACT

It is supposed that human pathogens, e.g. *Helicobacter pylori* abuse lipid raft domains on the host cell plasma membrane to infect the cell. Investigating DRM-associated molecules we identified the transmembrane adapter proteins (TRAPs), non-T cell activation linker (NTAL) and lymphocyte-specific protein tyrosine kinase (Lck)-interacting membrane protein (LIME) to be regulated by *H. pylori* in the human epithelial cell line HCA-7. Up to now, raft-associated TRAPs were exclusively described to mediate signal propagation downstream of antigen receptors. Our results posed the question whether these proteins adopt a role in *H. pylori*-infected epithelial cells too. Our studies revealed that *H. pylori* induces tyrosine phosphorylation of NTAL as well as LIME within 15 min of infection. We observed that activated NTAL and LIME bind to the Src homology 2 (SH2)-domain of growth factor receptor-bound protein 2 (Grb2) within 15 to 30 min of infection and associate with the c-Met receptor. Further, NTAL has a contributory role in regulating *H. pylori*-induced extracellular signal-regulated kinase (ERK) activation. After suppression of NTAL protein levels by siRNA, ERK phosphorylation was reduced to approximately 50%. Additionally, the knockdown of NTAL suppressed the phosphorylation of cytosolic phospholipase A_2 ($cPLA_2$). Activated $cPLA_2$ catalyzes the release of arachidonic acid (AA), whose metabolites are pivotal mediators in the *H. pylori*-induced inflammatory response. Thus, we propose that NTAL participates in the activation of the c-Met-Grb2-ERK-$cPLA_2$ signalling cascade at early stages of *H. pylori* infection.

1. Introduction

The gram-negative, microaerophilic bacterium *Helicobacter pylori* is one of the most successful human microbial pathogens [1]. It persistently colonizes the stomach of half the world's population increasing the risk of peptic ulcer disease, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphomas [1]. The *cag* pathogenicity island (PAI), a cluster of up to 28 genes, is one of the best characterized virulence factors. It encodes a type IV secretion system (T4SS), that translocates cytotoxin associated gene A protein (CagA), muropeptides and possibly other molecules into epithelial host cells [2]. *H. pylori* is attracted to lipid rafts by sensing a cholesterol gradient [3]. Contact with lipid rafts was shown to be required for the *H. pylori* virulence factor vacuolating toxin (VacA) [4] to enter the cell and for the T4SS-mediated translocation of CagA [5].

Numerous studies have demonstrated that pathogens abuse lipid raft domains in the host cell plasma membrane as concentration devices and signalling platforms [6]. Lipid rafts are liquid-ordered (lo) phase microdomains proposed to exist in biological membranes. They have been widely studied by isolating detergent-resistant membrane microdomains (DRMs). Although DRMs isolated from cells do not correspond precisely to pre-existing rafts in living cells, enrichment of a protein in DRM fractions indicates that it is raftophilic and tends to associate with rafts when they form [7].

In hematopoietic cells, lipid raft-associated transmembrane adapter proteins (TRAPs) are involved in modulating signal transduction by recruiting Src homology 2 (SH2)-domain containing cytosolic molecules to the cell membrane via multiple tyrosine-

Abbreviations: TRAP, transmembrane adapter protein; NTAL, non-T cell activation linker; LIME, lymphocyte-specific protein tyrosine kinase (Lck)-interacting membrane protein; LAT, linker for activation of T cells; PAG, protein associated with glycosphingolipid-enriched microdomains; SH2, Src homology 2; Grb2, growth factor receptor-bound protein 2; ERK, extracellular signal-regulated kinase; $cPLA_2$, cytosolic phospholipase A_2; AA, arachidonic acid; PAI, *cag* pathogenicity island; T4SS, type IV secretion system; Cag A, cytotoxin associated gene A protein; VacA, vacuolating toxin; SFK, Src-family kinase; Csk, carboxyl-terminal Src kinase; HGF, hepatocyte growth factor; EGFR, epidermal growth factor receptor; siRNA, small interfering RNA; DRM, detergent-resistant membrane microdomain; PBMC, peripheric blood mononuclear cell; COX-2, cyclooxygenase-2; PGE_2, prostaglandin E_2.

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based signalling motifs. TRAPs are substrates of Src-family kinases (SFKs). Four raft-associated TRAPs have been identified: linker for activation of T cells (LAT), protein associated with glycosphingolipid-enriched microdomains (PAG), non-T cell activation linker (NTAL) and lymphocyte-specific protein tyrosine kinase (Lck)-interacting membrane protein (LIME). Activated PAG and LIME are capable of recruiting the catalytic SFK inhibitor carboxyl-terminal Src kinase (Csk) to the membrane [8].

Among the lipid raft-associated TRAPs LAT, NTAL and LIME are implicated in the assembly of growth factor receptor-bound protein 2 (Grb2)-containing complexes stimulating extracellular signal-regulated kinase (ERK) activation in different hematopoietic cell types [9–11]. Grb2 binds to the specific phosphopeptide motif pYXNX via its central SH2-domain. TRAPs may recruit Grb2 from the cytosol to the membrane and thereby deliver Grb2-bound Son of sevenless homolog 1 (Sos1) in proximity to the small GTPase Ras, which then activates ERK [8].

So far TRAPs have not been described to be involved in tyrosine kinase receptor signalling in epithelial cells. However, Grb2 is known to associate with tyrosine kinase receptors, including hepatocyte growth factor (HGF) receptor (c-Met). HGF treatment of epithelial cells induces ERK phosphorylation [12]. It was shown by Churin et al. [13] that *H. pylori* activates c-Met independent of the PAI. ERK is also activated by *H. pylori* in a PAI-independent manner, but PAI-positive strains induce moderately stronger ERK phosphorylation [14]. In addition to c-Met, other receptors, e.g. epidermal growth factor receptor (EGFR) [15] or human EGFR2 (Her2/Neu) [13], are capable to induce ERK activation.

In the present study we analysed lipid raft-associated TRAPs in *H. pylori*-infected human epithelial cells. Herein, we provide evidence that NTAL and LIME are regulated by *H. pylori*. An inducible interaction of NTAL and LIME with the Grb2-SH2-domain as well as with the c-Met receptor is demonstrated. Furthermore, we show that NTAL exerts a functional role in *H. pylori*-induced phosphorylation of ERK and $cPLA_2$.

2. Materials and methods

2.1. Materials

The following antibodies were used to detect the respective proteins: Caveolin (Abcam, Cambridge, UK), Clathrin Heavy Chain, PTP1D/SHP2 (BD Biosciences, San Jose, CA, USA), EGFR 1005, ERK, Flotillin1, Grb2 E-1, GST, LIME-06, Met C-28, phospho-Tyr99, phospho-Tyr20 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), phospho-$cPLA_2$ (Ser^{505}), $cPLA_2$, phospho-ERK1/2, phospho-Met ($Tyr^{1234/1235}$), NTAL/LAB, phospho-Tyr100 (Cell Signalling Technology Inc., Danvers, MA, USA), NAP-08 (V. Horejsi, Prague, Czech Republic), phospho-Tyr4G10 (Upstate/Millipore, Schwalbach, Germany), T7-tag (Novagen, Merck Chemicals Ltd., Beeston, UK). EGF was purchased from Sigma (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), HGF from Calbiochem (Merck Chemicals Ltd., Beeston, UK) and tumour necrosis factor (TNF)α from R&D Systems (Wiesbaden-Nordenstadt, Germany). Glutathione S-transferase (GST) fusion proteins of Grb2-SH2 were prepared and used as described previously by Grabbe and Wienands [16].

2.2. Cell culture and transfection

The epithelial human colon carcinoma cell line HCA-7 (European Collection of Cell Cultures, Salisbury, UK) was cultured in RPMI 1640 complete medium (PAA Laboratories, Cölbe, Germany) containing 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) in a humidified 5% CO_2 atmosphere.

For transfection cells were cultured free of serum and antibiotics in either 60 mm- or 100 mm-dishes and were transfected with expression plasmids using Effectene Transfection Reagent (Qiagen, Hilden, Germany) or with siRNA using siLentFect™ Lipid Reagent (Bio-Rad, Munich, Germany) according to the manufacturer's instructions.

2.3. Plasmid construction

Expression plasmids coding for NTAL or LIME respectively were constructed by cloning the sequences into the eukaryotic expression vector pcDNA3 (invitrogen, Paisley, UK). In addition, the T7- and the His_6-tag sequences were included in the pcDNA3 vector at the 5′ end of the MCS.

2.4. Small interfering RNA

Knockdown of NTAL and LIME expression was achieved by transfection of cells with custom-synthesized small interfering RNA (siRNA) duplexes. The target sequences were as follows: 5′-GGUG-CAAAGAGGUCAGAGA-3′ (NTAL) or 5′-GGGUCUGCAAGCCUAAAAG-3′ (LIME). Scrambled siRNA (siNeg) was used as a negative control.

2.5. RNA isolation, reverse transcription-PCR and quantitative real time-PCR

Total RNA was extracted with RNeasy Mini Kit (Qiagen). cDNA was synthesized from 2 µg of RNA using oligo$(dT)_{18}$ primers, dNTPs, Ribonuclease inhibitor (Fermentas, St. Leon-Rot, Germany) and AMV Reverse Transcriptase (Promega, Mannheim, Germany). For quantitative real time-PCR primers (0.25 µM), fluorescein (1: 1×10^5, Bio-Rad) and SensiMix (Quantace, Berlin, Germany) were added in a dilution of 1: 20 to the cDNA mixture. Reactions were performed in the iCycler (Bio-Rad) using the following protocol: denaturation (95 °C for 10 min), amplification (40 cycles: 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s), melting curve program (72–95 °C; 0.5 °C), and cooling to 22 °C.

2.6. H. pylori cultivation and infection

The *H. pylori* strain P1 wild type (wt), its isogenic mutant strains *cagA* and *virB7* lacking a functional T4SS [17] as well as *H. pylori* P14 possessing an inactivated *vac*A gene (kindly provided by R. Haas, Max von Pettenkofer-Institute for Hygiene and Medical Microbiology, Munich, Germany) were cultured on agar plates containing 10% horse serum for 48–72 h in a microaerophilic atmosphere (generated by Campy Gen, Oxoid, Basingstoke, UK) at 37 °C. HCA-7 cells were grown in complete medium to reach 60% of confluency. 16 h before infection, the medium was replaced by fresh RPMI 1640 medium. For infection, the cell monolayer (60–80% confluence) was incubated with the bacteria at a multiplicity of infection (MOI) of 100 for different periods of time.

2.7. Preparation of detergent-resistant membrane microdomain (DRM)-fractions

All steps were conducted at 4 °C. $0.8–1\times10^8$ cells washed in PBS were lysed in 0.5 ml TNE buffer (50 mM Tris, 100 mM NaCl, 5 mM EDTA) supplemented with 1 mM Na_3VO_4, 50 mM NaF, 10 mM $Na_4P_2O_7$ and 1 mM AEBSF in the presence of 3% Brij-58 (Pierce/Perbio Science, Bonn, Germany). Cell lysates were homogenized with 10 strokes of a Potter-Elvehjem homogenizer, mixed with an equal volume of 80% sucrose (w/v) in MNE (25 mM MES, 150 mM NaCl and 5 mM EDTA) and placed in Beckmann ultracentrifuge tubes. The samples were immediately overlaid with 1 ml 30% sucrose and 0.5 ml 5% sucrose (w/v) in MNE. The gradient was centrifuged for 20 h at 200.000 g at 4 °C. Membrane domains were harvested by collecting 5 fractions of 250 µl (R1–R5) and 4 fractions of 310 µl (F1–F4) from the top of the gradient.