CagA of *Helicobacter pylori* alters the expression and cellular distribution of host proteins involved in cell signaling

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**Abstract**

To expand our knowledge of *Helicobacter pylori* virulence mechanisms, we used iTRAQ (isobaric tagging reagents for relative and absolute quantification)-based proteomic analysis to investigate the effect of *H. pylori* on gastric AGS tissue culture cells. In particular, we were interested in finding out which effects of *H. pylori* were dependent on the cytotoxins CagA and VacA. Protein analysis was restricted to detergent-resistant membranes (DRMs), because both toxins were described previously to localize in lipid raft-like domains. Using *H. pylori* wild type and two isogenic mutants, ΔcagA and ΔvacA, we identified a total of 21 proteins that were either increased or decreased in the DRMs due to bacterial infection. The effect on three of these proteins, ezrin, syndecan-4 and Rab11-FIP1, were furthermore dependent on CagA. Because these proteins have been implicated in cell migration, adhesion and polarity, they might act as important mediators of CagA cytotoxicity.

**Introduction**

*Helicobacter pylori* is a human gastric pathogen that is associated with severe gastric diseases including active gastritis, peptic ulcer disease and gastric adenocarcinoma (Peek & Blaser, 2002; Sepulveda & Graham, 2002). Two cytotoxins of *H. pylori* play a major role in disease development. CagA is translocated into host cell membrane via a type IV secretion system (T4SS) (Segal et al., 1999; Asahi et al., 2000; Backert et al., 2000; Odenbreit et al., 2000; Stein et al., 2000) and has been implicated in increased inflammation (Brandt et al., 2005; Shibata et al., 2006), cell proliferation and cell migration (Ricci et al., 1996). CagA was also shown to interfere with epithelial cell integrity by disrupting tight (Amieva et al., 2003; Bagnoli et al., 2005) and adherence junctions (Murata-Kamiya et al., 2007). Opening of tight junctions occurs through CagA-mediated binding and redistribution of the scaffolding protein ZO-1 and junctional adhesion molecule 1 (JAM-1). These effects are further enhanced by the activity of the second major cytotoxin of *H. pylori*, the vacuolating toxin VacA, which opens tight junctions by a yet unidentified mechanism (Papini et al., 1998). VacA additionally causes vacuolization of late endosomal compartments and apoptosis via the mitochondria-dependent pathway (Cover & Blanke, 2005).

We recently performed iTRAQ (isobaric tagging reagents for relative and absolute quantification) labeling and mass spectrometry (LC-MS-MS) analysis of detergent-resistant membranes (DRMs) of AGS cells infected with *H. pylori* wild type or noninfected and discovered that CagA inhibits the serine/threonine-kinase Par-1 (MARK2), which results in polarity defects and inhibition of tubulogenesis, a process crucial for proper cell differentiation (Saadat et al., 2007; Zeaiter et al., 2008). Encouraged by these results, we performed further iTRAQ/LC-MS-MS studies to explore the effects of *H. pylori* on AGS cells in more detail. This time, we additionally included the isogenic mutants ΔcagA and ΔvacA in the analysis to reveal new insights into the function of both toxins. This analysis allowed us to identify three proteins, ezrin, syndecan-4 and Rab11-FIP1, which showed differential association with host cell membranes and DRMs in a CagA-dependent manner. We suggest that CagA may modify these proteins to alter cell proliferation, motility and membrane recycling pathways, which may ultimately contribute to cellular transformation.
Materials and methods

Bacterial strains and growth conditions

*Helicobacter pylori* strain G27 and its isogenic mutants Δ*cagA*, Δ*virB9* (ΔHPS528) and Δ*vacA* were used in this study (Stein et al., 2002). Strains were cultured on brucella broth agar plates or a medium supplemented with selective antibiotics and 10% fetal bovine serum (FBS) and incubated at 37 °C for 48 h in an anaerobic jar containing a Campygen gas pack (5% O₂, 10% CO₂ and 85% N₂) (Oxoid). Cultures were incubated with 165 r.p.m. rotation at 37 °C overnight.

Cell culture

The human gastric adenocarcinoma cell line AGS was obtained from ATCC (ATCC# CRL-1739) and cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS (Invitrogen). Cells were maintained at 37 °C in 5% CO₂ atmosphere and 95% humidity.

Antibodies, dot- and immunoblotting

For immunoblotting, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) (GE Healthcare) membranes. PVDF and nitrocellulose membranes were blocked for 1 h with protein-free T20 blocking buffer (Pierce), washed and incubated with primary antibodies overnight at 4 °C. The following primary antibodies were used: anti-Flotillin 1 (BD Transduction Laboratories), anti-Rab11-FIP1 (Novus Biologicals), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (e-biosciences), anti-CD71 (3B82A1), anti-Lyn (H-6), antiezrin and antis Syndecan-4 (all from Santa Cruz). Horseradish peroxidase-conjugated secondary antibodies (GE healthcare) were used together with the Super Signal kit (Pierce) and Hyperfilm-ECL (GE healthcare) to visualize proteins. Protein intensities were normalized to those of GAPDH. Protein intensities were determined using the ImageQuant 400 imager and the IMAGEQUANT TL software (GE Healthcare).

Cell fractionation

AGS cells were grown, infected, washed, scraped and precipitated as described previously (Zeaiter et al., 2008). Approximately 9 × 10⁶ AGS cells were resuspended in 150 μL of saponin buffer [50 mM Tris/Cl, pH 7.5, 1 mM PMSF, 1 mM Na₃VO₄, 1 mM NaF, plus a cocktail of protease inhibitors (complete, EDTA-free, Roche), 1% w/v saponin] and incubated on ice for 10 min. Samples were pelleted (16 100 g for 5 min) and the supernatant was transferred to a new tube (cytosol fractions). The cells were resuspended a second time with 800 μL of saponin buffer and then pelleted again. The supernatants were discarded and 150 μL of lysis buffer [saponin buffer +1% Triton X-100 (v/v)] was added to solubilize cell membranes. Lysates were incubated on ice for 10 min, pelleted to remove the Triton X-100 insoluble fraction and supernatants were transferred to new tubes (membrane fraction). Twenty-five microliters of 5 × Laemmli sample buffer (+5% β-mercaptoethanol) was added to each of the cytosolic and membrane fractions and samples were boiled for 10 min. For total cell lysates, 9 × 10⁶ AGS cells were washed, scraped and precipitated as described. Precipitates were resuspended in 400 μL of phosphate-buffered saline, 80 μL of 5 × Laemmli sample buffer was added, and cells lysates were boiled for 10 min.

AGS infection and DRM preparation

AGS cells (6–8 × 10⁵) were washed twice using antibiotic-free RPMI medium containing 5% FBS, pH 6.5. One part of AGS cells was infected with *H. pylori* strains G27, G27Δ*cagA* and G27Δ*vacA* for 4 h at 37 °C. The humming bird phenotype was verified by microscopy. DRMs were prepared as described previously (Zeaiter et al., 2008).

iTRAQ-based proteomic analysis

Reducing of proteins, blocking, digestion, iTRAQ-labeling (Applied Biosystems) and two-dimensional LC-MS-MS analysis were carried out as described previously (Zeaiter et al., 2008). For data analysis, the PRO QUANT SOFTWARE v1.1 (Applied Biosystems) was used to identify and quantify proteins resolved by LC/MS/MS. iTRAQ peptide data were used to search the Celera Discovery System (CDS; v. Rev C) FASTA Files (Applied Biosystems) and the Swiss-Prot database (v. March 05; ftp://ftp.ncbi.nih.gov/blast/db/FASTA/). PRO QUANT-generated results were analyzed using the PROTEIN PILOT software v. 1 (Applied Biosystems). Fold change by ±1.25 was considered significant.

Statistics

The SIGMASTAT 3.5 software was used. Statistical analysis was carried out using a one-way ANOVA followed by the Tukey post hoc test for multiple comparisons. Significance was set at *P* < 0.05.

Results

Identification of DRM proteins

Because the two *H. pylori* cytotoxins, CagA and VacA, were identified previously in DRMs of host epithelial cells (Schraw et al., 2002; Asahi et al., 2003; Nakayama et al., 2006; Lai et al., 2008), we decided to determine the protein composition of DRMs of AGS cells following infection with *H. pylori* wild-type strain G27 or alternatively with the isogenic mutants Δ*cagA* or Δ*vacA*. Following the infection...
of AGS cells for 4 h, the cells were harvested, solubilized with Triton-X100 buffer and DRMs were isolated on sucrose density gradients. Sucrose gradient fractions were tested by immunoblotting for the presence of DRM markers flotillin-1 and Lyn and for the non-DRM marker transferrin receptor (CD71). Fractions 4 and 5 were identified as DRMs (Fig. 1) and were used for iTRAQ-based proteomic analysis.

**CagA mediates an increase in syndecan-4 and ezrin and a decrease in Rab11-FIP1 in the DRMs**

Using the advantages of iTRAQ labeling and LC-MS-MS, we were able to perform a relative quantification of changes in protein composition that occurred during infection with the different strains. The experiment was performed in duplicate and a total of 21 proteins were commonly identified with a significant P-value in both experiments (Table 1). Eleven of these proteins were detected under all four conditions (AGS/+G27/+ΔcagA/+ΔvacA), five were detected for (AGS/+G27/+ΔcagA) and five for (AGS/+G27/+ΔvacA). The differential recruitment of keratin-9, keratin-18, syndecan-4, ezrin and Rab11 family-interacting protein 1 (Rab11-FIP1) to the DRMs was significantly dependent on the presence of CagA (Table 1). Compared with wild-type-infected cells, the DRMs of cells infected with the vacA mutant did not show a significant difference in the level of recruitment for any protein (Table 1).

Because syndecan-4, ezrin and Rab11-FIP1 are implicated in pathways that are known targets of CagA (Lock & Stow, 2005; Morgan et al., 2007; Prag et al., 2007), we decided to

*Table 1. Proteins identified in the DRMs*

<table>
<thead>
<tr>
<th>Gene (Uniprot accession #)</th>
<th>Protein description</th>
<th># of query*</th>
<th>G27/AGS</th>
<th>ΔcagA/AGS</th>
<th>ΔvacA/AGS</th>
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<tr>
<td>RPL6 (O14548)</td>
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<td>–1.79</td>
<td>–1.46</td>
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<td>KRT18 (P05783)</td>
<td>Keratin, type I cytoskeletal 18</td>
<td>13</td>
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<td>MYH9 (P35579)</td>
<td>Myosin-9</td>
<td>36</td>
<td>–1.56</td>
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<td>LGALS3 (P17931)</td>
<td>Galectin-3</td>
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<td>–1.50</td>
<td>–1.30</td>
<td>–1.67</td>
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<tr>
<td>MYO1C (O00159)</td>
<td>Myosin-ic</td>
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<td>–1.38</td>
<td>–1.18</td>
<td>–1.62</td>
</tr>
<tr>
<td>COX7A2L (O14548)</td>
<td>Cytochrome c oxidase subunit V1a-related protein, mitochondrial precursor</td>
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<td>–1.13</td>
<td>–1.18</td>
<td>–1.14</td>
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<td>FLOT1 (O75955)</td>
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<td>1.11</td>
<td>–1.05</td>
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<td>RPN1 (P04843)</td>
<td>Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1</td>
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<td>+1.03</td>
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<td>KTN1 (Q86UP2)</td>
<td>Kinecin (kinesin receptor)</td>
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<td>+1.62</td>
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<td>+1.57</td>
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<td>SDC4 (P31431)</td>
<td>Syndecan-4</td>
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<td>+2.27</td>
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<tr>
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<td>+1.24</td>
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<td>EZR (P15311)</td>
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<td>IARS (P412S2)</td>
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<td>–1.46</td>
<td>ND</td>
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<tr>
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<td>8</td>
<td>–1.28</td>
<td>ND</td>
<td>–1.56</td>
</tr>
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<td>MARK2 (Q7KZ17)</td>
<td>Serine/threonine-protein kinase MARK2</td>
<td>3</td>
<td>+6.65</td>
<td>ND</td>
<td>+6.84</td>
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</table>

*Listed proteins had at least two recovered peptides with 99% confidence interval, P < 0.05 for quantification. ND, not identified (proteins had only one peptide recovered). Fold change by ± 1.25 was considered significant. Proteins that were modified dependent on CagA are shown in bold.*

**Fig. 1.** Preparation and identification of DRM fractions (indicated by the dashed box) by flotation assay. One-microliter fractions were collected from the top of the gradient (1–12).
investigate these proteins in more detail. MS/MS spectra of the iTRAQ reporter ions of peptides of these three proteins affected by CagA were consistent with values depicted in Table 1 (Supporting Information, Figs S1–S3). Information concerning mass accuracy, identification score and individual iTRAQ ratios for each peptide is provided in Table S1.

**CagA mediates cellular redistribution of syndecan-4, ezrin and Rab11-FIP1**

We further investigated whether *H. pylori* affected the expression and cellular distribution of these proteins and whether this process was CagA dependent. AGS cells were infected with wild-type G27 or ΔcagA for 4 h. Total cell lysate, cytosolic and membrane fractions were prepared and tested for the presence of each of the three proteins.

Both strains, wild type and ΔcagA, significantly increase the total and cytosolic amount of syndecan-4 in infected AGS cells by two- and 1.6-fold, respectively at 4 h postinfection (Fig. 2a and b). This indicates that CagA was not involved in this increase. However, syndecan-4 recruitment to AGS cell membranes was partially CagA dependent. *Helicobacter pylori* wild type increased membrane association of syndecan-4 threefold whereas the ΔcagA strain increased it only 1.6-fold. This difference between the G27 and the ΔcagA strain was significant.

*Helicobacter pylori* strain G27 significantly increased the total amount of ezrin c. 1.5-fold, whereas the cagA mutant seemed to reduce it a factor of 1.3 (Fig. 3a). The cytosolic amount of ezrin was increased 2.5-fold upon infection with wild type, but no increase was recorded for the infection with the ΔcagA mutant (Fig. 3b). Membrane fractions analysis showed a 1.5-fold increase of ezrin in wild-type-infected cells, whereas infection with the ΔcagA mutant reduced ezrin c. 1.6-fold (Fig. 3c). These results indicate that CagA mediated the redistribution of ezrin to the cell membrane.

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Fig. 2. Effects of *Helicobacter pylori* strains on syndecan-4 abundance and redistribution. AGS cells were infected by wild-type and ΔcagA strains and total cell lysate, cytosolic and membrane fractions were tested for the presence and amount of syndecan-4 by Western blot. Blots are representative of three independent experiments. Bars represent SD among three independent experiments. *P < 0.05 and **P < 0.001.
Rab11-FIP1 was reduced by a factor of 3 and 1.7 in the total AGS cell lysate and cytosolic fractions upon infection with G27 wild type, respectively, whereas the mutant strain ΔcagA increased the amount of Rab11-FIP1 approximately twofold in these fractions (Fig. 4a and b). In the membrane fractions of wild-type-infected AGS cells, Rab11-FIP1 was reduced by a factor of 2.1 but the amount of protein was not significantly modified by ΔcagA infection (Fig. 4c). These data indicate that the partial exclusion of Rab11-FIP1 from the cell membrane upon *H. pylori* infection is CagA dependent.

Similar results were obtained when we infected AGS cells with the T4SS mutant ΔvirB9 (ΔHP528), indicating that CagA translocation was necessary for the cellular redistribution of these three proteins (Fig. S4).

**Discussion**

The CagA and VacA cytotoxins of *H. pylori* are two important virulence factors that were shown to reduce matrix adhesion (Amieva *et al.*, 2003; Suzuki *et al.*, 2005), to disrupt intercellular junctions (Amieva *et al.*, 2003) and to induce cell motility (Snider *et al.*, 2008). Here we used iTRAQ labeling and LC-MS-MS proteomic analysis of DRMs of AGS cells infected with *H. pylori* wild type or alternatively the cagA and vacA isogenic mutants. Our aim was to potentially identify new cellular targets of one or both cytotoxins. The iTRAQ-based proteomic data revealed 21 host proteins to be increased or decreased in the DRMs during infection. The association of three of these proteins, ezrin, syndecan-4 and Rab11-FIP1, with the DRMs was CagA dependent. Furthermore, cell fractionation experiments demonstrated that CagA was also necessary for the redistribution of ezrin and syndecan-4 from the cytosol to the host membrane. The opposite effect was observed for Rab11-FIP1, which showed a CagA-dependent decrease in the host membrane.

Ezrin is a member of the ERM (ezrin–radixin–moesin) family of proteins that localize in the cell cortex comprised of the plasma membrane and the underlying cytoskeleton...
Hughes & Fehon, 2007). In polarized cells, ezrin is highly enriched in the microvilli at the apical side of the cells, where it links filamentous actin to various cell membrane proteins. This important feature of ezrin explains its role in various processes including membrane transport, cell morphology, cell adhesion and motility. A previous report indicated that *H. pylori* upregulated ezrin expression twofold in AGS tissue culture cells (Lim et al., 2004). Another study showed that ezrin is dephosphorylated on tyrosine residues as a consequence of CagA-mediated inhibition of Src-kinases (Selbach et al., 2004). The authors suggested that ezrin dephosphorylation might play a role in *H. pylori* attachment and/or invasion. How this relates to CagA-mediated toxicity remained unclear. Detergent resistance is a characteristic of membrane-rafts and of many raft-associated proteins, although it has become clear that detergent resistance is not a sufficient criterion for raft association. Nevertheless, our results imply that ezrin expression is not only increased following infection with *H. pylori*, but that ezrin increasingly associates with specialized raft-like microdomains in the host cell membrane and that this process is CagA dependent.

Based on the study by Selbach et al. (2004), it is also conceivable that ezrin dephosphorylation on tyrosine is a driving factor for the redistribution of ezrin to the DRM fraction. In fact, activated ezrin was shown to recruit Cdc42 to lipid rafts (Prag et al., 2007) and raft recruitment was required for Cdc42 activation and induction of cell migration. Therefore, we suggest that CagA causes recruitment of ezrin to the host cell membrane and its accumulation and activation in membrane microdomains and this may induce cell migration and contribute to the CagA-dependent invasive phenotype.

Syndecan-4, a ubiquitous transmembrane proteoglycan, is enriched in focal adhesions of adherent cells. It is not only implied in cell adhesion but also in cell proliferation and migration in response to growth factors (Morgan et al., 2007). Recently, Kwok et al. (2007) have demonstrated that the T4SS pilus adhesion CagL binds to α5β1-integrins. Interestingly, activation of α5β1-integrins is sufficient for cell spreading, but not for full formation of vinculin-containing focal adhesions, which requires costimulation of syndecan-4 with a heparin-binding fragment.
Smith et al. (2006) recently reported that syndecan-4 expression is induced by H. pylori in MKN45 cells in an NF-κB-dependent manner. Our data showed similar results in that total and cytosolic amounts of syndecan-4 increased upon infection of AGS cells with H. pylori independently of the CagA status. On the other hand, our observation that syndecan-4 is increased in the host cell membranes and DRMs during infection with wild type but not with the cagA mutant may indicate that CagA-dependent recruitment of syndecan-4 is crucial to control cell adhesion and cell migration in synergy with z5β1-integrins (Morgan et al., 2007).

Rab11a is a small GTPase that is involved in intracellular vesicle trafficking in the apical region of the polarized cell, but was also shown to mediate trafficking of E-cadherin to the adherens junction (Goldenring et al., 1996; Ulrich et al., 1996; Lock & Stow, 2005). This function was suggested to be important for the development of cell polarity. Rab11a is regulated by interaction with various Rab11-binding proteins including Rab11-FIP1, 2, 3, 4 and 5, and interaction of Rab11a with each binding partner may be important in separate signaling processes (Jin & Goldenring, 2006). Rab11-FIB1 has also been implicated in plasma membrane recycling (Jin & Goldenring, 2006). The CagA-dependent reduction of Rab11-FIP1 in the DRMs and host cell membrane fractions during infection may indicate that CagA negatively affects the recruitment of active Rab11 to the plasma membrane as well as to trafficking vesicles and this process may interfere with regular apical recycling and development of cell polarity.

In summary, iTRAQ labeling and LC-MS-MS proved to be a useful technique to gain new insights into H. pylori-mediated signaling pathways. Although we did not succeed in identifying VacA-dependent signaling events, we identified three molecules, ezrin, syndecan-4 and Rab11-FIP1, which were affected during infection of AGS cells in a CagA-dependent fashion. Further investigation into the specific role of these molecules may provide important clues to better understand as to how H. pylori CagA regulates these molecules to subvert host cell functions.

Acknowledgements

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References


Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Identification and quantification of a representative Syndecan-4 digest.

Fig. S2. Identification and quantification of a representative Ezrin digest.

Fig. S3. Identification and quantification of a representative RAB11FIP1 digest.

Fig. S4. ΔcagA and ΔvirB9 (HP528) show similar effects on expression and redistribution of ezrin, syndecan-4 and Rab11–FP1.

Table S1. Identified peptides of the 3 significantly regulated proteins.

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