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Role of the *cag* pathogenicity island encoded type IV secretion system in *Helicobacter pylori* pathogenesis

Nicole Tegtmeyer[1], **Silja Wessler**[2], and **Steffen Backert**[1,3]

[1]University College Dublin, School of Biomolecular and Biomedical Sciences, Science Center West, Belfield Campus, Dublin-4, Ireland [2]Paris-Lodron University of Salzburg, Department of Molecular Biology, Division of Microbiology, Billroth Str. 11, A-5020 Salzburg, Austria [3]Otto von Guericke University Magdeburg, Institute for Medical Microbiology, Leipziger Strasse 44, D-39120 Magdeburg, Germany

Abstract

Helicobacter pylori is a very successful human-specific bacterium worldwide. Infections of the stomach with this pathogen can induce pathologies including chronic gastritis, peptic ulcers and even gastric cancer. Highly virulent *H. pylori* strains encode the *cag* (cytotoxin-associated genes) pathogenicity island which expresses a type IV secretion system (T4SS). This T4SS forms a syringe-like pilus structure for the injection of virulence factors such as the CagA effector protein into host target cells. This is achieved by a number of T4SS proteins including CagI, CagL, CagY and CagA which by itself bind the host cell integrin member β_1 followed by delivery of CagA across the host cell membrane. A role of CagA interaction with phosphatidylserine has also been shown to be important for the injection process. After delivery, CagA becomes phosphorylated by oncogenic tyrosine kinases and mimics a host cell factor for the activation or inactivation of some specific intracellular signaling pathways. Here we review recent progress in characterizing CagA-dependent and CagA-independent signalling capabilities of the T4SS which include the induction of membrane dynamics, disruption of cell-to-cell junctions, actin-cytoskeletal rearrangements as well as pro-inflammatory, cell cycle-related and anti-apoptotic transcriptional responses. The contribution of these signalling pathways to pathogenesis during *H. pylori* infections is discussed.

Keywords

Helicobacter pylori; signalling; type IV secretion; VirB5; VirB10

Introduction

Helicobacter pylori colonizes the surface area of the gastric mucosa in the human stomach and is one of the most adapted microbial pathogens. About 50% of the world's population carries this bacterium, causing chronic, often asymptomatic gastritis in all infected humans,

Corresponding author: Steffen Backert PhD, University College Dublin, Belfield Campus, School of Biomolecular and Biomedical Science, Science Center West, Dublin-4, Ireland, steffen.backert@ucd.ie, Tel: +353 +1 - 716 2155, Fax: +353 +1 - 716 1183.

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and more severe gastric diseases in up to 10–15% of infected people depending on the geographical region [1-3]. Infections commonly occur in early childhood and, if not treated by antimicrobial therapy, *H. pylori* can persist lifelong. Although *H. pylori* infections are often diagnosed with a pronounced cellular inflammation status, which is triggered by the host innate and adaptive immune systems, the bacteria are commonly not eliminated. Numerous mechanisms of immune evasion have been reported and *H. pylori* became a paradigm for chronic infections. Studies of *H. pylori* have revealed not only its ability to colonize individual hosts for many decades, but also that this bacterium has co-existed with humans for a very long period through history. Genetic studies indicate that *H. pylori* spread during human migrations from east Africa more than 60,000 years ago [4]. Based on this long time of co-evolution, there are some indications that colonization by *H. pylori* could have been beneficial for their human carriers and this probably provided selective advantages [3]. In the modern world, however, *H. pylori* infections can cause a heavy burden of morbidity and mortality as a consequence of peptic ulcer disease, mucosa-associated lymphoid tissue (MALT) lymphoma and, the most dangerous complication, gastric adenocarcinoma [1-3]. Gastric adenocarcinoma is the second leading cause of cancer-related death in the world and approximately 649,000 people die from this malignancy each year [1].

The clinical outcome of *H. pylori* infections is determined by highly complex host-pathogen interactions. Disease progression is constrained by various parameters such as the bacterial genotype, environmental determinants and genetic predisposition of the host. For example, specific polymorphisms in human genes with crucial functions in immunoregulatory and pro-inflammatory signaling such as interleukin-1β (IL-1β), Nod (nucleotide oligomerization domain), tumour necrosis factor alpha (TNF-α) or interleukin-8 (IL-8) have been associated with an increased risk of developing disease including gastric cancer as summarised in excellent review articles [1-3,5; for more details see Glossary file Doc. S1 in the Supplementary Material]. During the last two decades, the cellular and molecular mechanisms acquired by *H. pylori* to undermine host defences have been investigated intensively (Fig. 1). *H. pylori* isolates are surprisingly diverse both in their genome sequences and pathogenicity. Dozens of bacterial factors have been identified to influence the pathogenesis of *H. pylori*. There are two classical secreted virulence factors present in *H. pylori*, the vacuolating cytotoxin (VacA) and the CagA protein encoded by the *cag* (cytotoxin-associated genes) pathogenicity island (*cag*PAI). VacA interacts with numerous host surface receptor molecules and can trigger various responses including pore insertion into the cell membrane, modification of endo-lysosomal functions, cell vacuolation, apoptosis and immune inhibition [1-3]. Much research interest worldwide is also focused on CagA which is encoded by more virulent strains but is typically missing in less virulent *H. pylori* isolates. Thus, the protein has been recognized as a marker for the *cag*PAI locus [6,7]. Other well-known pathogenicity-associated processes include flagella-driven *H. pylori* motility, urease-triggered neutralization of pH, several adhesins (BabA/B, SabA, AlpA/B, HopZ, OipA and others) mediating binding of *H. pylori* to gastric epithelial cells, glycosylation of cholesterol by HP0421, cleavage of E-Cadherin-triggered opening of cell-to-cell junctions by the protease HtrA, downregulation of antimicrobial nitric oxide production by arginase RocF as well as γ-glutamyl transpeptidase (GGT) which inhibits T-cell proliferation and others as summarised in Fig. 1 [1-3,5,8]. In addition, *H. pylori* induces a pronounced pro-inflammatory phenotype in infected gastric epithelial cells by multiple signalling activities that stimulate the transcription factors NF-κB and/or AP-1 [5,9]. There are also numerous other reported marker genes for *H. pylori*-induced disease development (e.g. *iceA* and *dupA*), but their biological function is widely unclear. Here we review the various *cag*PAI functions and multiple host cell signalling pathways with emphasis on their potential role in the pathogenesis of *H. pylori*.

The *cag* pathogenicity island encodes a type IV secretion system: two pilus assembly models

Intensive research in recent years has demonstrated that the *cag*PAI encodes functional components of a type IV secretion system (T4SS). This T4SS represents a needle-like structure (also called T4SS pilus) protruding from the bacterial surface and is induced by host cell contact to inject virulence factors [10,11]. T4SS transporters are commonly found in many Gram-negative bacteria and are evolutionary related to DNA conjugation machines [6]. The group of T4SSs is diverse both with respect to delivered substrates (DNA-protein complexes or proteins) and recipients, which can be either a bacterium of the same or other species, or organisms from a different kingdom (e.g. plants, fungi or mammalian cells). In addition to *H. pylori*, T4SSs have been found in *Agrobacterium*, *Bordetella*, *Bartonella*, *Legionella*, *Anaplasma* and other pathogens. T4SS transporters typically consist of 11 VirB proteins (encoded by *virB1-virB11* genes) and the so-called coupling protein (the NTPase VirD4). The prototypic and best characterised T4SS is the VirB or T-DNA transfer system of *Agrobacterium tumefaciens*. The agrobacterial VirB proteins can be grouped into three categories: (i) the core components or putative channel (VirB6-10), (ii) the pilus-associated components (VirB2, and possibly VirB3 and VirB5) and (iii) the energetic components (the NTPases: VirB4 and VirB11). VirB1 is an enzyme with muraminidase activity possibly enabling localized lysis of murein to achieve T4SS assembling at a given location [6]. In *Agrobacterium*, signal peptidase-I removes signal peptides from precursors of the main pilus component VirB2 and the minor pilus component VirB5, followed by cyclization of VirB2 by an unknown factor. Processed VirB2 and VirB5 subsequently associate with the membranes as stabilised by VirB4 and VirB8. Stabilized and properly oriented VirB5 then forms a complex with VirB2, which is a key step in the formation of the T4SS pilus assembly subcomplex. A model for individual steps in the assembly of the agrobacterial T4SS is summarised in Fig. 2A. When looking at the *H. pylori* T4SS, all orthologs of the 11 VirB proteins and VirD4 have been identified to be encoded in the *cag*PAI as well as some accessory factors [4,6] leading to a T4SS model similar to that of *Agrobacterium* (Fig. 2B). In line with these conclusions, immunogold-labelling studies indicated that the tips of the T4SS pilus are decorated with CagL [11], a proposed VirB5 ortholog [6]. In a second model (Fig. 2C), it was proposed that the T4SS appendages in *H. pylori* are covered locally or completely by CagY (VirB10) [10] and the model includes all identified VirB components, except VirB5 [12]. Interestingly, CagY is a very large protein (about 250 kDa) that contains two transmembrane domains with the mid domain (also called the repeat domain) exposed to the extracellular space [10]. Thus, it is still not fully clear if the *H. pylori* T4SS pilus is more similar to that of *Agrobacterium* which is mainly composed of VirB2 subunits and VirB5 or if it is composed of CagY as major pilus subunit or if it is a mix of both (Fig. 2B/C).

However, the only reported effector molecules injected by the *H. pylori* T4SS are peptidoglycan and CagA. Immunogold-stainings indicated the presence of CagA at the tips of T4SS pili, thus providing the first direct evidence that CagA might be delivered through these surface appendages, an observation which has not yet been reported for any other known T4SS effector protein in the bacterial world [11]. Investigation of the injection mechanism has shown that delivery of CagA requires a host cell receptor, the integrin member β_1 [11,13] and phosphatidylserine [14]. Numerous structural T4SS components have been demonstrated to bind to integrin β_1 including CagL [11], or CagA, CagI and CagY but excluding CagL [13]. However, while very little is known about CagA and CagI in the above context, CagL has been investigated in more detail. Like the human extracellular matrix protein fibronectin, CagL carries a RGD-motif shown to be important for interaction with integrin β_1 *in vitro* and on host cells, as well as downstream signaling to

activate the kinases FAK and Src [11], but mutation of the RGD-motif in CagL had no defect in T4SS functions such as phosphorylation of injected CagA during infection in another study [13]. These reports indicate that there is a controversy in the literature about the importance of the CagL RGD-motif in T4SS functions and host cell signaling. Another unsolved question is the proposed structure of CagY with respect to which domain is exposed to the extracellular space. The repeat domain in the middle of CagY has been shown to be accessible to labelling by antibodies made specifically against this subdomain [10]; however, yeast-two hybrid screens and other *in vitro* binding studies indicated that the very carboxy-terminus interacted with integrin β_1 [13] which has been proposed to be cytoplasmic [10] as shown in Fig. 2C. Thus, the role of the CagL RGD-motif and CagY topology for injection of CagA is not yet solved. It is also unclear why the effector protein CagA itself can bind to the extracellular domain of integrin β_1, because one would expect that such high binding affinity inhibits the injection process. However, these studies clearly showed that *H. pylori* targets integrin β_1 as a receptor for the T4SS, and deletion of *cagI*, *cagL* and *cagY* genes disrupt T4SS functions almost completely [11,13]. Thus, each of these factors exhibits important functional roles but their concerted interaction activities are unknown.

However, since a receptor is involved in host recognition by the T4SS it can be proposed that CagA is not injected into target cells at random positions but rather in a tightly controlled manner [15]. Importantly, integrins are normally not found at the apical membrane but at the basal membrane of polarized gastric epithelial cells (Fig. 1). This suggests the existence of a sophisticated control mechanism by which *H. pylori* injects CagA [11]. Basically, there are two major injection models which can be considered. First, *H. pylori* could inject its T4SS effectors across the basolateral membrane (Fig. 1). A possible scenario is that early exposed *cag*PAI-independent factors such as the *H. pylori* adhesins as well as HtrA, VacA, OipA and others may loosen intercellular epithelial junctions at locally restricted areas before a limited number of bacteria may gain access to integrins and inject CagA. The basal injection model of CagA can also explain why *H. pylori* does not cause more gastric damage in infected individuals and may only inject virulence proteins into target cells under specific conditions *in vivo*. Second, *cag*PAI-independent signaling events might stimulate the transcytosis of integrin molecules from the basal to the apical side of the cells, a process that has been suggested for integrin β_1 [15]. Indeed, disruption of host-cell polarity by another pathogen (enteropathogenic *Escherichia coli*) enabled basal membrane proteins to migrate apically. Transcytosis of integrins would therefore enable *H. pylori* an intriguing possibility to target the integrin β_1 receptor at apical membranes (Fig. 1). The latter scenario would explain how *H. pylori* T4SS substrates might be injected apically, possibly in part, to further disrupt intercellular junctions or activate early signaling events leading to the induction of pro-inflammatory genes, respectively.

Functional studies of *H. pylori* using animal infection models and transgenic mice

Recent functional studies in animal models have provided compelling evidence for the importance of CagA and *cag*PAI in *H. pylori* pathogenesis. Mongolian gerbils (*Meriones unguiculatus*), several knockout and other mice (e.g. INS-GAS mice) and Rhesus monkeys have been shown to serve as useful *in vivo* models to study *H. pylori*-induced pathology. However, each animal model has distinct advantages and disadvantages and can, therefore, be only considered as complementary systems. The most extensively studied model with respect to host inflammatory and physiological responses to *H. pylori* is the Mongolian gerbil [1,16-18]. Mongolian gerbils have been shown to develop similar *H. pylori*-induced pathology as compared to humans. *H. pylori* reproducibly induces gastric inflammation in this system and *cag*PAI-positive as well as various *H. pylori* mutants colonize gerbils

sufficiently well, which allow the examination of the role of bacterial virulence determinants in gastric injury. For example, gerbils were challenged by the *cag*PAI-positive strain TN2 and its isogenic mutants of *cagE* (*virB4*) or *vacA* for 62 weeks [16]. The wild-type and *vacA* mutants induced severe gastritis, whereas *cagE* mutants induced far milder changes. Gastric ulceration was induced at the highest rate (22/23) by wild-type *H. pylori*, followed by the *vacA* mutant (19/28). No ulcers were found in the gerbils infected with the *cagE* mutant (0/27) or in controls (0/27). Intestinal metaplasia was also found in the gerbils infected with the wild-type (14/23) or *vacA* mutant (15/28). Gastric cancer developed in one gerbil with wild-type infection and in one with *vacA* mutant infection [16]. These early data suggested that *cag*PAI-positive *H. pylori* can induce gastritis and gastric ulcer in gerbils with an important role of the T4SS. Further studies indicated that *H. pylori* strain B128 (also harbouring a functional *cag*PAI) increased plasma gastrin, a factor known to promote gastric epithelial hyperproliferation, but not infection with isogenic mutants lacking either *cagA* or *cagY* [17]. Enhanced corpus colonization with the parental wild-type strain was also evident. Virulence factors such as the *cag*PAI are therefore likely to impact on gastric physiological changes observed with *H. pylori* infection either directly, via permitting colonization of the corpus mucosa as a consequence of increased acid tolerance or indirectly, via promoting enhanced inflammation. Interestingly, infection of gerbils by *H. pylori* led to the development of inflammation-induced gastric adenocarcinoma in some but not all studies, highlighting the possible importance of different gerbil lines, diet, genetic differences between *H. pylori* strains and probably other parameters [1,17,18]. For example, gerbils infected with the *cag*PAI-positive strain 7.13, a gerbil-adapted derivative of B128, developed gastric dysplasia by only four weeks in 88% of gerbils, which was accompanied by adenocarcinoma in 25% of animals [18]. By eight weeks, gastric adenocarcinomas were present in 75% of infected gerbils that were sacrificed at this time-point and gastric adenocarcinomas were accompanied by severe lymphofollicular gastritis. Importantly, CagA and the T4SS played a crucial role in gastric cancer development of gerbils [18]. Consequently, further efforts have been put into identifying the mechanism of *H. pylori* -associated carcinogenesis. A first direct causal link between CagA and oncogenesis *in vivo* was identified by the generation of transgenic C57BL/6J mice expressing CagA in the absence of *H. pylori* [19]. CagA transgenic mice showed gastric epithelial hyperplasia and some mice developed gastric polyps and adenocarcinomas of the stomach and small intestine. Systemic expression of CagA further induced leukocytosis with IL-3/GM-CSF hypersensitivity and some mice developed myeloid leukemias and B cell lymphomas [19]. These results indicate that *H. pylori* can rapidly induce gastric adenocarcinoma in gerbils in a T4SS-dependent manner and that expression of CagA alone in transgenic mice is sufficient to induce severe malignant lesions. Therefore, it is clear that CagA and the T4SS play a central role in the pathogenesis of *H. pylori in vivo*.

H. pylori in vitro infection models: T4SS-dependent but CagA-independent cellular signalling

In addition to the above discussed *in vivo* models, the use of several *in vitro* cell culture systems has been very efficient for studying signalling cascades which are of relevance to *H. pylori* disease development. In particular, gastric epithelial and colonic cell lines (e.g. AGS, AZ-521, Caco2, HEp-2, KATO-III, MKN-28, MKN-45, NCI-N87 and others), primary gastric epithelial cells and professional phagocytes, including human polymorphonuclear leucocytes (PMNs) and human or murine macrophage cell lines (e.g. J774A.1, JoskM, RAW264.7, THP-1, U937 and others) have been utilized. In the following sections we will highlight some of these *in vitro* studies and begin with the T4SS-dependent but CagA-independent signaling pathways as summarised in Fig. 3A.

Very early experiments have shown that *H. pylori* can actively block its own uptake by professional phagocytes [20]. Vital *H. pylori* are necessary to block the phagocytic uptake, and *H. pylori* also abrogated the phagocytes' ability to engulf latex beads or adherent *Neisseria gonorrhoeae* bacteria as control. This antiphagocytic phenotype was dependent on a functional T4SS because isogenic *virB7* and *virB11* mutants abrogated this effect [20]. Interestingly, the actual factor involved was not CagA because isogenic *cagA* mutants also blocked phagocytosis. These data indicate that *H. pylori* expresses a yet unknown T4SS factor exhibiting antiphagocytic activity which may play an essential role in the immune escape of this persistent pathogen (Fig. 3A). However, the majority of studies were performed on the interaction of *H. pylori* with cultured gastric epithelial cells. For example, *H. pylori* was reported to change histone H3 phosphorylation by a T4SS-dependent but CagA-independent process (Fig. 3A). Infection with *cag*PAI-poitive *H. pylori* strains decreases H3 phosphorylation levels both at serine residue 10 and threonine residue 3 [21]. These observations were based on mitotic histone H3 kinases such as vaccinia-related kinase 1 (VRK1) and Aurora B which were not fully activated in infected cells, resulting in a transient *H. pylori* -induced pre-mitotic arrest [21]. Together, these results show that *H. pylori* subverts key cellular processes such as cell cycle progression by a yet unknown T4SS factor. In addition, the results of numerous reports indicated that structural components of the T4SS but not CagA itself were required for the induction of pro-inflammatory signaling including the activation of NF-κB and AP-1 (Fig. 3A). This suggested that the T4SS might inject factors in addition to CagA or that the T4SS itself triggers the effect. Despite intensive efforts including a systematic mutagenesis of all *cag*PAI genes, the hypothetical additional effector remained unknown for many years. Another possible candidate was *H. pylori* peptidoglycan which can be recognized by Nod1, an intracellular pathogen-recognition molecule [5]. These observations suggested that T4SS-dependent delivery of peptidoglycan is responsible for activation of Nod1→NF-κB-dependent pro-inflammatory responses such as secretion of IL-8 [5]. Interestingly, *cag*PAI-positive *H. pylori* can induce the NF-κB-dependent expression of AID (a DNA-editing enzyme) in host target cells, which resulted in the accumulation of mutations in the tumour suppressor protein TP53 [22]. Thus, induction of AID might be a mechanism whereby gene mutations could emerge during *H. pylori*-associated gastric carcinogenesis. However, the actual bacterial T4SS factor(s) and pathways which activate both transcription factors, NF-κB and AP-1, are highly controversial in the literature and still not fully clear [9].

Infection of gastric epithelial cells with *H. pylori* was also reported to profoundly activate numerous receptor tyrosine kinases (RTKs) in a T4SS-dependent fashion including the epidermal growth factor receptor EGFR [23,24], hepatocyte growth factor receptor c-Met [23] and Her2/Neu [23]. Studies on the downstream signalling indicated that each of these RTKs can activate the MAP kinase members MEK and Erk1/2 (Fig. 3A). However, while activation of EGFR has been shown to induce pro-inflammatory responses leading to the secretion of IL-8 [24], activation of c-Met (but not EGFR or Her2/Neu) was involved in cell scattering and motogenic responses of infected gastric epithelial cells [23]. Interestingly, the non-receptor tyrosine kinase c-Abl and the small Rho GTPases Rac1 and Cdc42 are also activated by a T4SS-dependent but CagA-independent mechanism and play a role in triggering the scattering and motility of infected gastric epithelial cells (Fig. 3A). However, the actual T4SS factor involved was also unclear for many years.

Recent *in vitro* studies showed a profound role of recombinant CagL in activation of EGFR, Her3/ErbB3, Src and Fak kinases in an RGD-dependent manner [25]. Investigation of the molecular mechanism how CagL can activate EGFR has demonstrated to involve ADAM17, a metalloprotease involved in catalyzing ectodomain shedding of receptor tyrosine kinase ligands. In non-stimulated cells, ADAM17 is normally in complex with the integrin member $\alpha_5\beta_1$ and inactive. During acute *H. pylori* infection, however, it was shown that CagL

dissociates ADAM17 from the integrins and activates ADAM17 (Fig. 3A). This was confirmed by infection with a Δ*cagL* deletion mutant, which is entirely defective in the latter response and by genetic complementation with the wild-type *cagL* gene or biochemical complementation by addition of extracellular CagL restoring this function. In addition, during integrin binding studies using intact host cells and immobilised CagL on petri dishes it was found that CagL mimics some important functions of human fibronectin [25]. Fibronectin is a 250-kDa eukaryotic protein containing an RGD-motif which plays crucial roles in promoting cell adhesion, migration, and intracellular signaling. It was shown that purified CagL alone can directly trigger intracellular signaling pathways upon contact with mammalian cells and can even complement the spreading defect of fibronectin$^{-/-}$ knock-out cells *in vitro* [25]. During interaction with various human and mouse cell lines, CagL mimics fibronectin in triggering cell spreading and focal adhesion formation. CagL-mediated activation of the above mentioned kinases was essential in these processes. Interestingly, fibronectin activates a similar range of tyrosine kinases but not Her3/ErbB3 [25]. These findings suggest that the VirB5 ortholog CagL does not only exhibit functional mimicry with fibronectin but is also capable of activating fibronectin-independent signaling events. Interestingly, when the purified repeat region 2 or the carboxy-terminus of CagY was immobilized on petri dishes, neither of these fragments could induce efficient cell spreading [25]. Remarkably however, when CagL was mixed with CagY, the repeat region 2 but not the integrin β_1-interacting carboxy-terminus [13] enhanced the CagL effect [25]. This finding suggests that the internal repeat region of CagY and CagL may act cooperatively, and that the carboxy-terminal interaction of CagY with integrin β1 has a different function, further confirming that the observed cell spreading effect is specific for CagL. If other *H. pylori* factors such as extracellularly added CagY, CagI or CagA can also trigger similar and/or other intracellular signalling pathways, and whether CagL-mediated activation of EGFR, Her3/ErbB3, Src and Fak contributes to the injection of CagA during *H. pylori* infection was not yet investigated and needs to be addressed in future studies.

Phosphorylation-dependent cell signaling of injected CagA

An important reason why CagA was actually discovered as an injected effector protein is the very early observation that it undergoes tyrosine-phosphorylation (CagA^{PY}) by the host cell kinases Src and Abl [15]. Site-directed mutagenesis and mass spectrometry of CagA in *H. pylori* or transfected CagA identified numerous phosphorylation sites as the Glu-Pro-Ile-Tyr-Ala (EPIYA)-motifs A, B, C and/or D [7,26]. In infected or transfected epithelial host cells, CagA^{PY} has then been shown to interact with the Src homology 2 (SH2) domains of numerous eukaryotic signalling proteins. The first detected binding partner of CagA^{PY} was the tyrosine phosphatase Shp2 (Fig. 3B). Since then, nine other host cell factors were also reported to interact with CagA in a phosphorylation-dependent fashion: the tyrosine phosphatases Shp1, the adaptor proteins Grb2, Grb7 and Crk, phosphoinositide-3-kinase (PI3K), Ras GTPase activating protein RasGAP, and as well as the tyrosine kinases Csk, Src and Abl [26]. Thus, CagA^{PY} appears to mimick a tyrosine-phosphorylated host cell protein and therefore seems to act as a kind of masterkey or picklock to highjack crucial signaling pathways in the host. The various CagA^{PY}-SH2 domain interactions play complex roles in *H. pylori*-induced actin-cytoskeletal rearrangements, scattering and elongation of infected host cells in culture as summarised in Fig. 3B.

Gastric epithelial cells infected with *H. pylori in vitro* start to migrate and acquire a morphology that has been originally described as the “hummingbird phenotype”. This phenotype results from two successive events: the induction of cell scattering and cell elongation. While induction of early cell motility mainly depends on a CagA-independent T4SS factor [23], cell elongation is clearly triggered by CagA^{PY} [6,7]. Transfection experiments demonstrated that the CagA^{PY}-Shp2 interaction stimulates the phosphatase

activity of Shp2 which contributes to cell elongation by activating the Rap1→B-Raf→Erk signalling cascade and by direct dephosphorylation and inactivation of focal adhesion kinase, FAK [7]. Further studies have indicated that the $CagA^{PY}$-induced cell elongation phenotype also involves tyrosine dephosphorylation of cortactin, vinculin and ezrin, three well-known actin-binding proteins [6]. The phosphatases involved in this scenario, however, remained unknown and does not require Shp2. Instead, $CagA^{PY}$ can inhibit Src activity both by direct interaction of both proteins or by binding of $CagA^{PY}$ to Csk, a negative regulator of Src [6,7]. Because Src is the primary kinase phosphorylating CagA in the EPIYA-motifs, inhibition of Src by $CagA^{PY}$ generates a classical negative feedback-loop that appears to control the amount of intracellular $CagA^{PY}$. Cortactin, ezrin and vinculin are all Src substrates, and Src inactivation causes dephosphorylation of these proteins and is crucial for triggering cell elongation [26]. In addition, interaction of $CagA^{PY}$ with CrkII/Abl and/or PI3K may also activate the small Rho GTPases Cdc42 and Rac1, binding of $CagA^{PY}$ to Grb2 or Shp2 may regulate proliferative and pro-inflammatory signalling *via* the MAP kinase pathway while interaction of $CagA^{PY}$ with Shp1 may downregulate the latter response (Fig. 3B). Finally, several additional binding partners were identified such as Grb7 and RasGAP (Fig. 3B). The potential function of these two factors in molecular pathogenesis, however, is yet unknown and needs to be investigated in future studies. Taken together, $CagA^{PY}$ interacts with a surprisingly high number of host proteins to induce signalling pathways involved in cell scattering, elongation and probably other phenotypes.

Phosphorylation-independent signalling of CagA

Remarkably, not all interactions of injected or transfected CagA depend on its tyrosine phosphorylation. Together, 12 cellular interaction partners of non-phosphorylated CagA have been identified [26]. These interactions have been reported to induce the disruption of cell-to-cell junctions, loss of cell polarity and induction of pro-inflammatory and mitogenic responses (Fig. 3C). The first detected interaction partner of non-phosphorylated CagA was the adapter protein Grb2, and Grb2 is the only host factor described to interact with both non-phosphorylated and additionally with phosphorylated EPIYA motifs as described above [26]. In particular, non-phosphorylated CagA was shown to interact with Grb2 both *in vitro* and *in vivo*, which provides a mechanism by which Grb2-associated Sos (son of sevenless, a guanine-exchange factor of the small GTPase Ras) is recruited to the plasma membrane (Fig. 3C). The CagA/Grb2/Sos complex can promote Ras-GTP formation, which in turn stimulates the Raf→Mek→Erk signalling cascade leading to cell scattering as well as activation of nuclear transcription factors involved in cell proliferation and expression of the anti-apoptotic myeloid cell leukaemia sequence-1 (MCL-1) protein [27]. In line with these findings, CagA was also shown to function as a mimetic of the eukaryotic Grb2-associated binder (Gab) adaptor protein in transgenic Drosophila which can further explain how CagA triggers this signalling cascade [7]. Interestingly, CagA can also interact with RUNX3, a tumor suppressor which is frequently inactivated in gastric cancer, by a novel identified WW domain in the amino-terminal region of CagA [28]. In particular, CagA induces the ubiquitination and degradation of RUNX3, thereby extinguishing its ability to inhibit the transcriptional activation of RUNX3 (Fig. 3C). Additional evidence for a role of CagA in manipulating nuclear responses came from whole-genome microarrays and functional studies investigating host cell gene expression after infection of target cells with wild-type *H. pylori*, isogenic *cagA* and *cag*PAI mutants as well as CagA transfection. For example, it was shown that under certain circumstances CagA can also induce the transcription factor NF-κB influencing the expression of multiple target genes such as IL-8 in a CagA phosphorylation-independent manner as discussed recently [26]. These data suggest the presence of distinct EPIYA-independent domains within CagA which play essential roles in protein targeting and alteration of host-cell transcription signalling pathways.

Another important consequence of phosphorylation-independent CagA interactions in polarised epithelial cells is the disruption of cell-to-cell junctions (Fig. 3C). In particular, tight and adherens junctions are essential for the integrity of the gastric epithelium [15]. CagA interferes with these intercellular junctions *via* several pathways. Injected CagA associates with the epithelial tight-junction scaffolding protein ZO-1 (zona occludens-1) and the transmembrane protein JAM (junctional adhesion molecule), causing an ectopic assembly of tight-junction components at sites of bacterial attachment [2]. Non-phosphorylated CagA was also reported to interact with the transmembrane cell-cell junction protein E-cadherin [7]. Later it was found that CagA forms a complex with c-Met recruiting E-cadherin and the Armadillo-domain protein p120 catenin indicating that the interaction between CagA and E-cadherin is not direct [29]. However, whether the 135 kDa c-Met receptor is phosphorylated and activated upon *H. pylori* infection is a matter of debate [26]. Thus, the role of c-Met in *H. pylori*–induced signalling is not fully clear and needs to be investigated more thoroughly in future. Controversy also exists whether CagA can induce disruption of the E-cadherin complex followed by the release of β-catenin, which has been proposed for transfected CagA or *H. pylori*-infected AGS cells [7,18]. There is some doubt if vector-based overexpression of CagA induces cellular effects which are really comparable to that of CagA injected by *H. pylori*. In addition, AGS cells do not express E-cadherin and show abnormal β-catenin distribution making them unsuited for the investigation of β-catenin signaling [29]. Emphasizing this aspect, using MDCK cells which express E-cadherin and β-catenin without any mutations, it was demonstrated that CagA is not involved in *H. pylori*-induced β-catenin signal transduction pathways during infection [30]. These data were also supported by several other research groups as recently shown.

However, the role of CagA in inducing the loss of cell polarity is much clearer. The kinase Par1b/MARK2 (partitioning-defective 1/microtubule affinity-regulating kinases), a central regulator of cell polarity, was found to play a role in *H. pylori*-induced signalling. Non-phosphorylated CagA can directly bind Par1b and inhibits its kinase activity to trigger the loss of cell polarity [7]. In addition, more recent studies also showed that CagA not only binds to Par1b but also to other members of this kinase family (including Par1a, Par1c and Par1d), and that these interactions contribute to the *H. pylori*-induced elongation phenotype of AGS cells. Taken together, these findings demonstrate that injected or transfected CagA can interfere with Par1, c-Met and E-cadherin signalling and may also activate NF-κB, thereby contributing to *H. pylori*-induced pro-inflammatory responses [9,26]. However, the downstream pathways of CagA appear very diverse and possible crosstalk among those and other bacterial factors needs to be dissected in more detail. Finally, there are two additionally reported binding partners of CagA, α-Pix and integrin β1 as mentioend above (Fig. 3C), but the functional importance of these interactions is yet unclear and also needs to be investigated in future studies.

Concluding remarks

H. pylori represents one of the most successful human pathogens, which induces severe clinical symptoms only in a small subset of individuals mirroring a highly balanced degree of co-evolution of *H. pylori* and humans. Studies of host-bacterial interactions and virulence factors CagA and the T4SS have provided us with many fundamental insights in processes leading to *H. pylori* pathogenesis. The number of more than twenty known cellular interaction partners of CagA is very astonishing and remarkable for a bacterial effector protein. The current hypothesis implies a model with translocated CagA as an ‘eukaryotic’ signaling mimetic molecule either present in a large multiprotein complex or simultaneously in separated locations of infected target cells, which may have important impact on the multi-step pathogenesis of *H. pylori*. The high number of binding partners also reflects the integrated network of complex signal transduction pathways in host cells, which is

coordinated through CagA itself or initiated by the T4SS-host interaction underlining their overall importance as observed in numerous *in vitro* and *in vivo* studies. In future, it will be important to search for additional injected proteins because it seems rather unlikely that the entire *cag*PAI was aquired during evolution to only inject one effector protein (CagA) and peptidoglycan. Interestingly, recent computational predictions suggested that proteins encoded by HP0522 and HP0535 maybe novel T4SS effectors [4]. Whether HP0522 and HP0535 are indeed translocated by the T4SS is only one open question among many others that need to be answered in future studies in order to uncover the complex mechanisms how *H. pylori* interacts with host cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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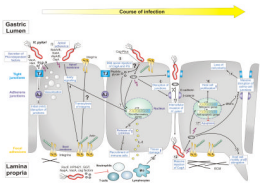

Figure 1.

Model of *Helicobacter pylori*-induced epithelial-barrier disruption and pathogenesis. The interplay between polarized gastric epithelial cells and a variety of bacterial pathogenicity factors modulates multiple host responses during the course of infection, as indicated. *H. pylori* expresses several adhesins such as BabA, BabB, SabA, AlpA and AlpB as well as OipA which mediate apical binding of the bacteria (1). Attached *H. pylori* or those in the mucus secrete virulence factors into the medium (HtrA protease, VacA cytotoxin and others) (2), which could trigger mild opening of tight junctions (TJs) and adherens junctions (AJs) at early time points of infection (3). While internalized VacA causes cellular vacuolization, a hallmark of the ulceration process, HtrA cleaves the junctional protein E-cadherin [8]. Another intriguing possibility of junction disruption could be the transcytosis of basal integrins to the apical surface by early, but unknown, *cag*PAI-independent signaling (4). Apical exposure of some integrin molecules such as integrin $\alpha_5\beta_1$ could stimulate the T4SS pilus to inject CagA and peptidoglycan into cells (5). Injected CagA can then be targeted to TJs and AJs followed by further disruption of these junctions (6). Injected CagA and peptidoglycan (5) in addition to OipA (1) can trigger nuclear factor-kB (NF-κB) activation (7) and the release of proinflammatory cytokines such as IL-8 (8). These cytokines can alter the secretion of mucus and induce changes in gastric-acid secretion and homeostasis. They also attract immune cells to infiltrate from the blood stream into the gastric mucosa (9), where they cause substantial tissue damage at the site of infection (10). *H. pylori* also express numerous factors to suppress immune cell functions as indicated. The result of the above described processes is local epithelial disruption enabling some *H. pylori* of entering the intercellular space between adjacent cells and reaching the basal membranes (11). In this manner, the bacteria could access integrins and inject CagA (12). Injected CagA can then induce the massive disruption of cell junctions (13) and loss of cell polarity (14). The induction of metalloproteases (MMPs) might enhance this effect in addition to HtrA. Finally, CagA can induce multiple pathways to trigger host-cell motility and elongation (15) and the onset of mitogenic genes and cell proliferation (16), and it can inhibit apoptosis (17). The interplay of each of these pathways could result in substantial deregulation and oncogenic transformation of gastric epithelial cells *in vivo* and, thus, are important for *H. pylori* pathogenesis. Specific steps labeled with question marks are untested or speculative aspects of the model. Other specific abbreviations used: α5β1 (chains of the integrin receptor), *cag* (cytotoxin-associated genes), ECM (extracellular matrix), IL-8 (interleukin 8), HP0421 (cholesterol-alpha-glucosyltransferase), MΦ (macrophage), NapA (neutrophil-activating protein A), PAI (pathogenicity island), PG (peptidoglycan), RocF (arginase enzyme). For more details see text and glossary in the Supplementary Material (Doc. S1). This model was updated from Wessler and Backert [15] with kind permission from Blackwell Publishing.

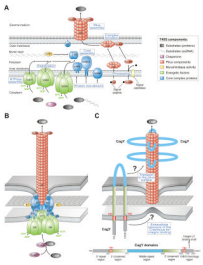

Figure 2.

Models for the assembly and assembled structure of T4SSs in *Agrobacterium tumefaciens* and *Helicobacter pylori*. (A) Proposed assembly of the prototypical *Agrobacterium* VirB/VirD4 T4SS machinery is shown. The T4SS is a multi-component protein complex spanning the inner and outer membranes of *Agrobacterium* and other Gram-negative bacteria. Current knowledge of T4SS assembly, cellular localization of its components is shown in a simplified manner. The coupling protein VirD4 and structural components (VirB1-VirB11) are typically required for secretion and are depicted according to their proposed functions. A model for T-pilus assembly in *Agrobacterium* showing the proposed VirB4-VirB8-VirB5-VirB2 interaction sequence leading to the formation of VirB2-VirB5 complexes followed by T-pilus assembly. The assembled T4SS then triggers the secretion of substrates from the bacterial cytoplasm directly into the cytoplasm of infected host cells or into the extracellular milieu. (B) Model-1 for the assembled T4SS machinery in *H. pylori* assuming that all VirB1-11 proteins are encoded by the *cag*PAI and assemble in a similar fashion as proposed for *A. tumefaciens* [6]. The reported substrates for this T4SS are CagA and peptidoglycan. (C) Model-2 proposes that the T4SS requires basically the same VirB proteins as in panel B with one major difference. The pilus surface is proposed to be covered with CagY molecules. In contrast to VirB10 in many T4SSs, *H. pylori* VirB10 (CagY) is a very large protein (ca. 250 kDa, domain structure and amino acid positions shown for CagY of strain 26695, accession number NP_207323.1) carrying two transmembrane domains (TM1 and TM2) to form a hairpin-loop structure in the membranes as depicted [10]. Immunogold labelling against the loop region in CagY indicated that this part of the protein is exposed to the extracellular space and is transported to the pilus surface by a yet unknown mechanism [10]. However, recent data demonstrated that the very carboxy-terminus of CagY can bind to the host receptor integrin β_1 [13]. How the latter domain can be exposed to the extracellular space in order to make contact with integrin β_1 is not yet clear and must be clarified in future studies.

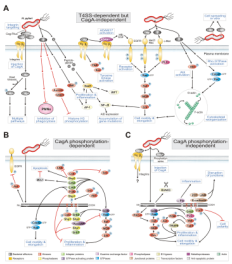

Figure 3.

Model for the role of *H. pylori* T4SS in host cell signaling processes which may effect pathogenesis. (A) The *H. pylori* T4SS pili are induced upon contact with host cells and can inject effector molecules such as the CagA protein and peptidoglycan in a manner dependent on integrin β_1. Injected CagA can then induce cascades as depicted in the panels below. Panel A highlights a multitude of known T4SS-dependent but CagA-independent pathways involved in the activation of receptor and non-receptor tyrosine kinases, pro-inflammatory signalling, Rho GTPase activation, scattering and motility of gastric epithelial cells as well as suppression of histone phosphorylation and *H. pylori* phagocytosis by immune cells. Two particular T4SS factors have been reported to be involved in some but not all of these responses. The known signaling functions for injected peptidoglycan as well as pilus-exposed or recombinant CagL are shown. For numerous other pathways the actual T4SS factor is yet unknown as also indicated. (B) CagA phosphorylation-dependent and (C) phosphorylation-independent signal transduction events. CagA is injected into the host cell membrane of infected gastric epithelial cells which also requires phosphatidylserine. The tyrosine kinases Src and Abl phosphorylate injected CagA. CagA can then modulate various signaling cascades associated with cell polarity, cell proliferation, actin-cytoskeletal rearrangements, cell elongation, disruption of tight and adherens junctions, pro-inflammatory responses and suppression of apoptosis, as depicted. Black arrows indicate activated sigalling pathways and red arrows correspond to inactivated cascades. Panels B and C were updated from Backert *et al.* [26] with kind permission from WILEY. For specific abbreviations and terms used in this figure, please see text and glossary in the Supplementary Material (Doc. S1).