CagI Is an Essential Component of the *Helicobacter pylori* Cag Type IV Secretion System and Forms a Complex with CagL

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**Abstract**

*Helicobacter pylori*, the causative agent of type B gastritis, peptic ulcers, gastric adenocarcinoma and MALT lymphoma, uses the Cag type IV secretion system to induce a strong proinflammatory response in the gastric mucosa and to inject its effector protein CagA into gastric cells. CagA translocation results in altered host cell gene expression profiles and cytoskeletal rearrangements, and it is considered as a major bacterial virulence trait. Recently, it has been shown that binding of the type IV secretion apparatus to integrin receptors on target cells is a crucial step in the translocation process. Several bacterial proteins, including the Cag-specific components CagL and CagI, have been involved in this interaction. Here, we have examined the localization and interactions of CagI in the bacterial cell. Since the *cagI* gene overlaps and is co-transcribed with the *cagL* gene, the role of CagI for type IV secretion system function has been difficult to assess, and conflicting results have been reported regarding its involvement in the proinflammatory response. Using a marker-free gene deletion approach and genetic complementation, we show now that CagI is an essential component of the Cag type IV secretion apparatus for both CagA translocation and interleukin-8 induction. CagI is distributed over soluble and membrane-associated pools and seems to be partly surface-exposed. Deletion of several genes encoding essential Cag components has an impact on protein levels of CagI and CagL, suggesting that both proteins require partial assembly of the secretion apparatus. Finally, we show by co-immunoprecipitation that CagI and CagL interact with each other. Taken together, our results indicate that CagI and CagL form a functional complex which is formed at a late stage of secretion apparatus assembly.

**Introduction**

The human gastric pathogen *H. pylori* is the principal cause of chronic active gastritis and peptic ulcer disease, and it is involved in development of gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma [1,2]. The molecular mechanisms leading to development of ulcers or cancer are not well-understood, but both host and bacterial factors are likely to contribute to disease development [3]. One of the major bacterial virulence factors is the *cag* (cytotoxin-associated gene) pathogenicity island, a 37 kb genomic island present only in a subset of *H. pylori* isolates and clearly associated with an enhanced risk of developing peptic ulcers or adenocarcinoma. The *cag* pathogenicity island encodes the Cag type IV secretion system, which induces secretion of chemokines such as interleukin-8 (IL-8) from gastric epithelial cells and is thus responsible for a strong proinflammatory response during infection, and the effector protein CagA which is translocated into epithelial and potentially other host cells. Several cellular interaction partners of CagA and associated changes in cellular functions have been described, but its exact function in the infection process is not clear. However, due to its correlation with the risk of cancer development [4], its role in pathogenesis in a Mongolian gerbil infection model [5], and direct evidence from transgenic mice [6], CagA is often considered as a bacterial oncoprotein [7].

Similar to other type IV secretion systems, components of the Cag system assemble into a multiprotein secretion apparatus spanning both bacterial membranes [8]. Since the Cag system contains proteins with common features or sequence similarities to most or all essential components of prototypical type IV secretion systems, such as the VirB system of *Agrobacterium tumefaciens* or plasmid conjugation systems, it is believed that the structure of the secretion apparatus and its molecular mechanisms of action are analogous. This implies the presence of a core complex bridging the cytoplasmic and outer membranes [9], of pilus-like structures at the bacterial cell surface [10,11], and of cytoplasmic or inner membrane-associated ATPases providing the energy for secretion apparatus assembly and protein transport. The pilus-like appendages on the bacterial surface seem to contain several apparatus proteins, but their exact composition has not been determined [10–12]. Interestingly, however, several proteins that have been shown or suspected to be part of these surface structures, interact with β1 integrins on the host cell surface. Proteins contributing to this interaction include the substrate CagA as well as the proteins...
CagL, CagY and CagL [13,14]. Whereas CagY has a C-terminal domain with sequence similarity to VirB10 family proteins and CagL, has been considered as a VirB5-like adhesin of the type IV secretion apparatus [15], CagI is a unique component without any sequence similarities to components of prototypical type IV systems, or to any other known protein. Although isogenic cagA mutants have been examined in several studies, there are conflicting reports about CagI requirement for type IV secretion system function. One study found that deletion of the cagl gene resulted in an impairment of CagA translocation, but not IL-8 induction [16], and two others found that the presence of cagl is required for IL-8 induction as well [17,18]. Since cagI is part of an operon containing several other genes involved in type IV secretion [19], and since none of these studies included complementation of a cagl mutant, the actual contribution of CagL to these phenotypes is not clear. However, a recent study which included complementation of a cagl mutant provided direct evidence that CagI is required for IL-8 induction [20].

In this study, we have constructed a set of cagl mutants with the aim of avoiding polar effects on expression of the essential downstream gene cagL, and we have complemented the mutants to gain insight into CagI function. Infection experiments show that CagI is an essential component of the Cag type IV secretion apparatus, required for both IL-8 induction and CagA translocation. We provide evidence that CagI is, at least partly, associated with the bacterial outer membrane, and exposed to the bacterial surface. Moreover, we show that it interacts with the putative integrin ligand CagL. These data suggest that both proteins may form a complex at the bacterial cell surface, and that this complex is required for productive interactions with target cells.

Results

Deletion of the Cagl gene Results in Low CagL Levels

There have been conflicting results concerning the requirement of Cagl for induction of IL-8 secretion from gastric epithelial cells in previous studies [16–18]. One reason for this discrepancy might be differences in the H. pylori strains used in each study, but it might also be due to subtle differences in gene expression patterns in the respective cagl mutants, given that cagl is part of an operon structure with several alternative transcriptional start sites [19]. Closer inspection of the cagl locus shows that the 3’ end of cagl overlaps with the start codon of cagL, and the Shine-Dalgarno sequence of cagl overlaps with the stop codon of cagH (Fig. 1A). Thus, cagl deletion and replacement by a resistance gene cassette may have polar effects on expression of the cagl gene. We sought to re-evaluate the contribution of cagl for type IV secretion system function by constructing cagl mutants devoid of such polar effects. To check for production of the Cagl protein, we raised an antiserum from whole cell lysates of strain P12 or its isogenic mutant (Fig. 2A), suggesting that insertion of the chloramphenicol resistance cassette in the cagl locus exerted a downstream transcriptional effect. Alternatively, it is possible that the absence of the Cagl protein influences CagL production and/or stability.

Since Cagl is essential for both CagA translocation and induction of IL-8 secretion [16], the role of Cagl for type IV secretion system function could thus not unambiguously be demonstrated with this mutant. To overcome this problem, we used a marker-free deletion procedure involving the streptomycin counter-selection system [21] to construct cagl deletion mutants. Since an alternative transcriptional start site for cagl has been identified in the 3’ region of the cagl gene [19], we constructed mutants in strain P12 in which the 5’ cagl part was either replaced by a streptomycin sensitivity/erythromycin resistance cassette (using plasmid pWS320), or deleted without insertion of a resistance marker (using plasmid pWS326; Fig. 1C). Western blot analysis with the polyclonal Cagl antiserum demonstrated that Cagl production was lost in these mutants, as expected, but cagl deletion again resulted in strongly reduced CagL protein levels (Fig. 2A, lanes 2 and 4). Complementation of the cagl mutants with a chromosomal (recA) integration vector containing the cagl gene under the control of the cagl promoter (plasmid pWS322; Fig. 1C) resulted not only in Cagl production, but also in restored CagL production (Fig. 2A; lanes 3 and 5). This suggested that the reduced CagL levels observed in the cagl mutants were not caused by transcriptional effects of resistance gene cassette insertion or gene deletion, but rather by direct effects of Cagl on CagL.

To corroborate this conclusion, we sought to quantify cagl transcript levels in these mutants. For this purpose, we prepared total RNA from liquid cultures of the different H. pylori strains, carried out reverse transcription into cDNA, and performed conventional as well as real-time PCR with cagl-specific primers and control primers specific for 16S-rRNA. In control PCRs, we obtained cagl-specific bands from cDNA, but not mRNA from P12 wild-type bacteria, confirming that the RNA preparation was not contaminated by genomic DNA (data not shown). Transcript quantification by qPCR showed no significant difference in cagl levels between wild-type bacteria and each cagl mutant (Fig. 2B), demonstrating that deletion of the 5’ cagl region did not interfere with transcription of the cagl gene.

Cagl is Required for CagA Translocation and IL-8 Induction

To examine the impact of cagl deletion on functionality of the Cag type IV secretion system, we performed infection experiments of AGS epithelial cells with H. pylori for 4 hours, and determined tyrosine phosphorylation of the CagA protein by Western blot analysis and induction of IL-8 secretion by sandwich ELISA. Infection experiments with the wild-type strain and the pWS320 and pWS326 mutants showed that cagl deletion results in a CagA translocation deficiency, as shown previously (Fig. 3A). In addition, these cagl mutants were unable to induce IL-8 secretion from AGS cells (Fig. 3B). Complementation of these mutants restored both CagA translocation and IL-8 induction after AGS cell infection (Figs. 3A and 3B).

While these results indicated an involvement of Cagl in both type IV secretion-associated phenotypes, it could still not be ruled out that the differing CagL levels are in fact responsible for these phenotypes. Therefore, we complemented the pWS320 cagl mutant with a chromosomal integration vector harboring the cagl gene under the control of the cagl promoter. The resulting strain produced an intermediate amount of CagL, but was nevertheless defective for CagA translocation and IL-8 induction (data not shown), suggesting that Cagl is independently required for type IV secretion system function. Furthermore, we generated a marker-free cagl deletion mutant (using plasmid pWS327; Fig. 1C) in which cagl was deleted in such a way that the cagl Shine-Dalgarno sequence is used as a ribosomal binding site for cagl, providing a “bypass” in the operon from cagl directly to cagl. Surprisingly, this cagl mutant produced normal levels of CagL (Fig. 2A), suggesting...
indicating that CagI is not absolutely required for CagL production or stability. However, quantification of cagL transcripts by qPCR showed a 6.04 ± 4.67-fold increase of cagL mRNA levels in the pWS327 mutant in comparison to wild-type bacteria (Fig. 2B), suggesting that precise deletion of the cagL gene resulted in a more effective transcription of the cagL gene, possibly via the cagF promoter. Interestingly, the cagI mutant obtained with plasmid pWS327 was also completely defective in both CagA translocation and IL-8 induction (Figs. 3A and 3B). Taken together, these results show that CagI is by itself an essential component of the Cag type IV secretion apparatus.

CagI and CagL Protein Levels in H. Pylori Cells Depend on the Presence of Various Secretion Apparatus Components

Mutual interdependence of protein levels is often found in multiprotein complexes such as type IV secretion machines, and has often been interpreted as stabilizing effects due to the presence of corresponding protein-protein interactions [22–25]. To determine if such effects occur between other Cag components and either CagI or CagL, we generated isogenic mutants of strain P12 in each cag gene known to have at least a partial function for Cag type IV secretion (Table 1), and we examined cell lysates of these mutants for CagI and CagL production by immunoblotting. As shown in Fig. 4, CagI production was influenced by the absence of several cag genes. Notably, the cagX, cagY, cagH and cagG mutants produced virtually no CagI, and several further mutants (Δcagδ, ΔcagW, ΔcagV, ΔcagU, ΔcagM, ΔcagI, and ΔcagE) produced significantly reduced amounts of CagI. In contrast, the cagF mutant produced normal levels of CagL. This suggests that inner membrane-associated structural components (CagU, CagV, CagW, CagY), components of a putative outer membrane-associated subcomplex (CagX, CagM, Cagδ), and also CagL, may have an impact on CagI stability. Interestingly, CagI levels were strongly reduced in the cagV and cagX mutants as well (Fig. 4), whereas control measurements by qPCR showed that the levels of cagL transcripts were not significantly different in these mutants (data not shown). Significant reductions of CagL levels were also found in the cagδ, cagW, cagI, cagH, cagG and cagE mutants, but not in other mutants showing reduced CagI levels (ΔcagV, ΔcagU, ΔcagM, and again not in the cagF mutant (Fig. 4).

CagI is Present in Different Subcellular Pools and is Partly Surface-exposed

To obtain evidence for CagI and CagL localization, we fractionated bacterial cells into soluble and membrane-associated
components, and extracted the total membrane fraction with 1% triton X-100, which preferentially extracts cytoplasmic membrane proteins [26]. In these fractionations, CagI was found both in the membrane fraction and in the soluble fraction containing cytoplasmic and periplasmic proteins (Fig. 5A). Membrane-associated CagI was not completely extracted by triton X-100, suggesting that CagI might be partly associated with the outer membrane. In contrast, the majority of CagL was found in the soluble fraction, and only a minor part in the total membrane fraction from where it was not extracted by triton X-100 (Fig. 5A). The distribution of CagL in the fractions was not significantly different in the wild type and the cagI mutant. Control immunoblots showed that the outer membrane-associated proteins CagX and AlpB were present only in the membrane fraction, and were not extracted by triton X-100, whereas the inner membrane-associated protein RecA was substantially extracted from the membrane fraction. As an independent method for membrane separation, we used isopycnic sucrose density gradient centrifugation of resuspended total membrane fractions. Immunoblot analysis of individual fractions from the sucrose gradients revealed two different membrane-associated pools of both CagI and CagL (Fig. 5B). Control immunoblots showed that only the high density fractions contained outer membrane-associated proteins, whereas the cytoplasmic membrane-associated protein RecA was present in several fractions.

While these results suggested the presence of separate CagI and CagL pools in different localizations in the bacterial cell, they did not reveal a clear association with the outer membrane or the bacterial surface. To obtain evidence for a possible localization at the surface, we performed a limited proteolytic digestion of intact bacterial cells with proteinase K. Protease accessibility of CagI and CagL was assessed by immunoblotting of equal amounts of untreated and proteinase K-treated cells. The amounts of both CagI and (to a lesser extent) CagL were significantly reduced by proteinase K treatment, whereas the cytoplasmic or cytoplasmic membrane-associated protein RecA was not digested, and the outer membrane protein AlpB was digested almost completely (Fig. 5C). Taken together, these data indicate that both CagI and CagL are partially localized at the bacterial surface, but that considerable pools of both proteins exist in a non-surface localization as well.

CagI Interacts with CagL

The influence of CagX, CagY and CagL on CagI protein levels, and of CagI on CagL protein levels, suggested that corresponding protein-protein interactions might exist. To determine whether

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**Figure 1. Operon organization of the cagH, cagI and cagL genes.** (A) Gene arrangement of the cag pathogenicity island in H. pylori strain P12. Genes are represented as arrows, and their designations are indicated below. The operon containing cagI may comprise five (cagF, cagG, cagH, cagI and cagL) or even more genes, but cagH, cagI and cagL are particularly tightly associated since they have overlapping reading frames or Shine-Dalgarno (SD) sequences. Translational starts are indicated by arrows. (B) A cagI deletion mutant containing a terminatorless chloramphenicol resistance cassette [16] was generated by transformation with plasmid pSO171. A polyclonal rabbit antiserum raised against CagI was used for immunoblot analysis of whole cell lysates of the P12 wild-type strain or isogenic cagA or cagI mutants. The same lysates were examined by Western blot for the presence of CagI using the polyclonal rabbit antiseraum AK271. CagI and CagL protein bands, respectively, are marked by arrowheads. Note that both antisera recognize cross-reactive bands, but anti-CagI does not react with CagL, or vice versa (data not shown). (C) Plasmid constructs used for generation and complementation of cagI mutants. A counterselection strategy was used for generating marker-free deletion mutants. Mutants generated with plasmids pWS320 and pWS326 retain the 3’ part of cagI, whereas the mutant generated with plasmid pWS327 has a complete deletion without any cagI traces. For complementation of cagI mutants in trans, the cagI gene was cloned under the control of the cagA promoter and integrated into the recA gene (plasmid pWS322).

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CagX and/or CagY interact with CagI, we performed immunoprecipitation experiments from *H. pylori* cell extracts with polyclonal CagX and CagY antisera, as described previously [24]. Western blot analysis of the precipitated proteins showed a co-precipitation of CagX with CagY and vice versa, but we were unable to detect CagI in the precipitation fractions (data not shown). To determine whether CagI interacts with CagL, we used the polyclonal CagL antiserum for immunoprecipitation experiments. Immunoblot analysis of the precipitated proteins showed that CagI was successfully precipitated, and that CagL was indeed co-precipitated with CagI (Fig. 6). In a control immunoprecipitation from the *cagI* mutant, CagL was not co-precipitated, demonstrating the specificity of the immunoprecipitation. To confirm this result, we performed a reverse immunoprecipitation using the polyclonal CagL antiserum AK271, which precipitated CagL together with a cross-reacting protein (Fig. 6). Consistent with the results described above, CagI was co-precipitated with CagL from an *H. pylori* P12 wild-type lysate. Again, immunoprecipitation from a lysate of the P12ΔcagL mutant did not result in precipitation of CagI. Taken together, these data demonstrate that CagI and CagL form a complex in *H. pylori* cells.

**Discussion**

Type IV secretion systems are known as a highly versatile group of macromolecule transporters [27,28], and this versatility is also
reflected in the variation of their respective components. Many well-characterized type IV secretion systems including some conjugation systems are composed of conserved essential components. In contrast, more divergent systems such as the Icm/Dot (type IVb) system of *Legionella pneumophila*, or the Cag system of *H. pylori*, usually include additional components with as yet unknown functions. The Cag system probably contains functional analogues to all VirB proteins and to VirD4 [8], but also additional essential components that are unique to this system. These additional components include two proteins (CagD/Cag3 and CagM) which have been shown to take part in an outer membrane-associated subcomplex [24,29], two predicted inner membrane proteins (CagU and CagH), and one protein (CagD) with a possible surface localization [30]. Using careful mutation and complementation studies, we show here that CagI is another essential component of the Cag type IV secretion apparatus. Furthermore, we show that CagI interacts with CagL, an essential Cag secretion apparatus component that, like CagI, has been shown to bind to integrin receptors on the target cell surface [13]. A recent study, which also included complementation of *cagI* mutants [20], reached the same conclusions.

The chromosomal region containing the *cagI* gene seems to be organized as an operon of five genes (*cagF, cagG, cagH, cagI* and *cagL*) [19], with the latter three open reading frames overlapping each other, indicating that there is a tight coupling of *cagH, cagI* and *cagL* gene expression. Although we found effects of *cagG* and *cagH* deletion on CagI and CagL protein levels, and of *cagI* deletion alone on CagL protein levels, *cagF* deletion did not seem to have much influence on the levels of either protein, arguing against simple transcriptional effects. This is also supported by our *cagL* transcript quantification data showing only minor differences between the wild-type strain and different *cagI* mutants, and by complementation of the *cagI* deletion mutants which restored CagL production without changing *cagL* transcript levels. Although we cannot exclude that translation of *cagL* transcripts or other post-transcriptional processes are less effective in the *cagI* deletion mutants, it is likely that the presence of the CagI protein and its interaction with CagL accounts at least partly for maintenance of CagL wild-type levels. A surprising finding was that the complete marker-free deletion of *cagI* (using plasmid pWS327) did not influence CagI protein levels. Since *cagL* transcript levels were

Figure 4. Presence of several Cag components influences CagI and CagL protein levels. Whole cell lysates of equal amounts of the wild-type strain P12 and of isogenic mutants in single cag genes (Table 1) were separated by SDS-PAGE and examined by immunoblotting with the anti-CagI and anti-CagL antisera, respectively. Representative immunoblots are shown. Note that the *cagI* mutant shown here was generated with plasmid pWS327. Arrowheads indicate the positions of CagI and CagL protein bands, respectively.
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Figure 5. Localization of CagI and CagL in bacterial cell fractions. (A) *H. pylori* cells grown in liquid culture for 24–48 hours were lysed by ultrasonication and subsequently fractionated into soluble and insoluble proteins by ultracentrifugation. Ultracentrifugation pellets containing membrane-associated and other insoluble proteins were extracted with 1% triton X-100 to separate outer membrane-associated (TX100 insol) from inner membrane-associated (TX100 sol) proteins. Comparable amounts of each fraction were analysed by immunoblot for their CagI and CagL content. As controls, immunoblots against the outer membrane-associated proteins CagX and AlpB, and the partly soluble and partly inner membrane-associated protein RecA were used. Representative immunoblots are shown. CagI and CagL bands are indicated by arrowheads. (B) Ultracentrifugation pellets were resuspended and subjected to isopycnic density gradient centrifugation on 25–60% sucrose gradients. Fractions were collected from the gradients and analyzed by immunoblotting with the indicated antiserum. CagI and CagL bands are indicated by arrowheads. (C) Bacteria were subjected to limited proteolytic digestion by proteinase K. Equal amounts of untreated control cells (P12) and proteinase K-treated bacteria (P12 PK) were analyzed by immunoblot with the indicated antisera.
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increased in this mutant, the most likely explanation is that a combination of higher cagL transcription and increased CagL degradation due to the lacking interaction with CagI resulted in almost wild-type levels of the CagL protein. It is not clear which effect was responsible for the differing phenotype reported previously for the cagL mutant in strain 26695 [16], but reconstruction of 26695 cagL deletion mutants resulted in the same phenotypes as described here (data not shown), arguing against strain-specific differences.

It is reasonable to assume that the genetic organization of cagG, cagH, CagI and CagL also reflects a functional connection between the gene products. Previous yeast two-hybrid screening procedures [25,31] detected interactions between CagI and several other Cag proteins including CagG, but none of them was confirmed except an interaction between recombinant CagI and CagZ fusion proteins in vitro [31]. We show here that the native CagI and CagL proteins take part in a protein complex in H. pylori cells, as also found in a recent study where immunoprecipitation of CagL from H. pylori lysates resulted in coprecipitation of CagI and CagH [20]. CagG, CagH, CagI, and CagL are all acidic proteins with predicted isoelectric points between 4 and 6. CagG, CagI and CagL contain N-terminal signal sequences and would thus be supposed to be transported to the periplasm, from where the latter two might reach the bacterial surface, as would be expected for integrin ligands. In contrast, CagH is most likely an integral cytoplasmic membrane protein, according to topology predictions [24]. An interesting feature of CagI is that its C-terminal amino acids (SKIVVK) are almost identical to the C-terminal amino acids of CagL (SKIVJK) and CagH (TKIVK); the latter two proteins also show a weak overall sequence similarity between each other (data not shown). Intriguingly, these C-terminal motifs have been shown to contribute to the function of these proteins [20], raising the possibility that they represent binding motifs for a common interaction partner of all three proteins.

Given that CagI and CagL were found to interact by co-immunoprecipitation, it is worth noting that the two proteins were not distributed equally in bacterial cell fractions. The majority of CagL was found in a soluble fraction, which also contained CagI, but more CagL seemed to be associated with the membrane fractions under the conditions used. Due to their N-terminal signal sequences, the most likely localization of the soluble pools of both proteins is in the periplasm. The membrane-associated pools of CagI and CagL showed a similar distribution in sucrose density gradient fractions, although Cagl was more readily extracted from a total membrane fraction with triton X-100. However, as described previously [24,26], it is not possible to completely separate cytoplasmic and outer membrane proteins of H. pylori using such standard procedures. Therefore, a clear assignment of the membrane pools to cytoplasmic membrane or outer membrane-derived vesicles, respectively, is not possible at this point, but the protease digestion experiments strongly suggest a partial surface exposition of the CagI protein. CagI showed a higher susceptibility for surface proteinase K digestion than CagL, but since CagL has been localized on surface appendages formed by the Cag secretion apparatus [13] and probably functions as a pilus-associated adhesion binding to integrin receptors [15], we would expect that CagL is also surface-exposed. It is not clear at this point whether CagI localizes to type IV secretion apparatus pili as well, and if so, whether the interaction between CagI and CagL takes place there, or if there is only an indirect interaction, mediated by a common interaction partner of both proteins. Unfortunately, possibly due to the cross-reactions of the CagI antiserum, we have so far been unable to localize CagI at the bacterial surface by immunofluorescence. Thus, it remains to be shown whether CagI is really associated with the pilus-like type IV surface appendages.

The decreased CagI levels in mutants lacking components of the putative type IV secretion apparatus core complex (ΔcagV, ΔcagW, ΔcagX, ΔcagY, and possibly ΔcagE), or in mutants lacking components of the putative outer membrane-associated subcomplex (ΔcagD, ΔcagM, and ΔcagX), might be taken as an indication that corresponding protein-protein interactions exist. However, since we were unable to detect any interaction between CagI (or CagL) and CagX or CagY by immunoprecipitation (data not shown), an alternative possibility is that wild-type CagI levels reflect correct protein localization, implying that CagI is transported by the secretion apparatus itself. In this scenario, an incompletely assembled secretion apparatus would result in failure of CagI to reach its destination, to form the putative CagI-CagL complex, and, as a consequence, in protein degradation. CagI and CagL would thus represent “late” components in secretion apparatus assembly, an assumption that is also supported by the observation that type IV secretion pili are not produced in the absence of CagI or CagL [20]. Further studies are required to elucidate the order in which the different Cag proteins assemble into a functional secretion or translocation complex.

In conclusion, we have demonstrated that CagI is an essential component of the Cag type IV secretion apparatus. Dependence of CagI protein levels on multiple cag genes and its interaction with CagL suggest that these two proteins take part in a subcomplex at the bacterial surface which might, possibly together with further components such as CagY, tether the secretion apparatus to integrin receptors to initiate translocation of CagA into host cells.
Materials and Methods

Bacterial Strains, Cell Lines and Transformation

*H. pylori* strains were grown on GC agar plates (Difco) supplemented with vitamin mix (1%), horse serum (9%), vancomycin (10 mg/l), trimethoprim (5 mg/l), and nystatin (1 mg/l) (serum plates), and incubated for 16 to 60 h in a microaerobic atmosphere (85% N₂, 10% CO₂, 5% O₂) at (1 mg/l) (serum plates), and incubated for 16 to 60 h in a microaerobic atmosphere (85% N₂, 10% CO₂, 5% O₂) at

37°C. *E. coli* strains Top10 (Invitrogen) and DH5α (BRL) were grown on Luria-Bertani (LB) agar plates or in LB liquid medium [32] supplemented with ampicillin (100 mg/l), chloramphenicol (30 mg/l), or kanamycin (40 mg/l), as appropriate. AGS epithelial cells (obtained from ATCC, number CRL-1759) were cultivated under standard conditions as described previously [33]. For the generation of isogenic mutants in *H. pylori* strain P12, the corresponding plasmids (Table 1) were introduced by natural transformation, as described [34]. *H. pylori* transformants were selected on serum agar plates containing 6 mg/l chloramphenicol or 8 mg/l kanamycin.

Plasmid Constructions

Standard cloning and DNA analysis procedures were performed according to [32]. Plasmid DNA was purified from *E. coli* by the boiling procedure and *E. coli* cells for electroporation were prepared according to the protocol recommended for the Gene Pulser (BioRad). Amplification of DNA fragments by polymerase chain reaction (PCR) was performed as described [34]. The *cagI* deletion plasmids pWS230, pWS232, and pWS236 were based on inverse PCR amplification of a gene library plasmid used for sequencing of strain P12 [35] with primers WS418 (5'-ACCGGTCGAC TAAAAAACAT TTCATATCTC-3') and WS419 (5'-ACCGGTCGAC GATGAGGAAA GAGATGTG-3'). The PCR product, which contained thus *cagI* flanking regions and the pSMART-HCKan cloning vector, was either digested with *SalI* and religated for plasmid pWS230, or digested with *BamHI* and *SalI* and subsequently ligated with a chloramphenicol resistance cassette to obtain plasmid pWS235, or with an *psL-em* cassette to obtain plasmid pWS230. The precise deletion construct pWS237 was obtained by inverse PCR from the same gene library plasmid using the 5'-phosphorylated primers WS420 (5'-CATATCTCTT TTCTCATTG-3') and WS421 (5'-AAAAAAGCTCG TAAAAATAC C-3'), and blunt-end religation of the PCR product. For complementation of the *cagI* mutants, plasmid pWS232 was constructed by PCR amplification of the *cagI* gene using primers WS31 (5'-GGACTAGTGA AGTGAGGAAA GAGATGTG-3') and WS308 (5'-ACCGGTCGAG TCATTTGACA ATAACCTTAG-3'), and cloning the PCR product via *BglII* and *XhoI* into pWS241 [36], a derivative of the chromosomal *recI* location. Expression of cloned genes under the control of the *cagI* promoter.

Antiseras and Immunoblotting

The antiserum against CagI was generated by immunization of a rabbit with a synthetic peptide comprised of 19 C-terminal amino acids of CagI (amino acids 350-368; H₂N-(C)NLEKRADL-WEEQLKLERET-COOH) coupled to KLH, as described previously [24]. Rabbit polyclonal antiserum against CagL, CagX, CagA, RecA, and AlpB have been described previously [10,24,38]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting was performed as described [16]. For the development of immunoblots, polyvinylidene difluoride (PVDF) filters were blocked with 5% non-fat milk powder in TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl), 0.1% Tween 20, and incubated with the respective antiseras at a dilution of 1:1000-1:5000. Alkaline phosphatase-conjugated protein A or horseradish peroxidase-conjugated anti-rabbit IgG antiserum was used to visualize bound antibody.

Tyrosine Phosphorylation Assay and Determination of IL-8 Secretion

Standard infections of AGS cells with *H. pylori* strains and subsequent preparations for phosphotyrosine immunoblotting were performed as described previously [33]. Briefly, cells were infected with bacteria at a multiplicity of infection of 100 for 4 h at 37°C, washed three times and suspended in PBS containing 1 mM EDTA, 1 mM Na₃VO₄, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin. Cells with adherent bacteria were collected by centrifugation and resuspended in sample solution. Tyrosine-phosphorylated proteins were analyzed by immunoblotting with the phosphotyrosine antiserum PY99 (Santa Cruz Biotechnologies). The production of IL-8 by AGS cells after infection with *H. pylori* strains for 4h was determined from cell supernatants by a sandwich ELISA as described [16].

RNA Isolation

Total RNA was isolated from *H. pylori* strains grown in liquid cultures for 16–24 h by the hot phenol method [19]. Briefly, bacterial cells were mixed with a stop solution containing 95% ethanol and 5% phenol and collected by centrifugation. Pellets were resuspended in 600 μl TE (pH 8.0) containing 0.5 mg/ml lysozyme; suspensions were mixed with 60 μl 10% SDS, incubated at 64°C for 2 min, mixed with 66 μl 1 M sodium acetate (pH 5.2), and extracted with 750 μl phenol at 64°C. Traces of phenol were precipitated from the aqueous phases with ethanol/sodium acetate (pH 6.5), redissolved in diethylypyrocarbonate-treated water, and treated with RNase-free DNase I (Fermentas). Total RNA was analyzed on nondenaturing 1.0% agarose gels and quantified on a NanoDrop ND 1000 spectrophotometer (Thermo Scientific).

cDNA Synthesis and qPCR

For cDNA synthesis, we used the First Strand cDNA Synthesis Kit (Fermentas) with 1–5 μg total RNA and random hexamer primers. Reverse transcription was carried out at 25°C for 5 min and at 37°C for 60 min, and the reaction was terminated by heating to 70°C for 5 min. Primers for qPCR were designed to amplify 90 bp to 150 bp regions of the *cagL* gene as well as 16S-rRNA (internal control). Reaction mixtures containing a master mix with SYBR green (Invitrogen, Hamburg, Germany) and each set of primers were added to a 96-well plate together with diluted cDNA samples at a final volume of 25 μl per well. Nuclease-free water was used as negative control. Samples were incubated in an ABI-Prism SDS7000 (Applied Biosystems, Darmstadt, Germany) for 43 cycles (30 s at 95°C, 30 s at 59°C, and 30 s at 72°C). Transcript levels were quantified by the comparative CT (cycle threshold) method and normalized to 16S-rRNA levels in each sample. The relative abundance of each transcript was calculated using the 2⁻ΔΔCT formula.

Bacterial Cell Fractionation

Bacterial cells were fractionated as described previously [26], with minor modifications. Briefly, *H. pylori* cells were grown in Brucella broth for 24–48 h, then harvested, washed and
concentrated by precipitation as above.

to the membrane suspension to a final concentration of 1%

were collected from the density gradients, and proteins were

triethanolamine buffer were layered on top of the gradients, and

EDTA) upon one another in SW41 rotor tubes, followed by

density gradients were prepared by layering solutions containing

ultracentrifugation (45 min, 230000

added to the membrane suspension to a final concentration of 1%

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refsuspended in precipitation buffer (10 mM Tris-HCl, pH 8.0, 1 mM PMSF, 1 μM leupeptin, 1 μM pepstatin). Bacteria were lysed by ultrasonication, and the lysate was centrifuged for 10 min at 7000 × g to remove unbroken cells and cell debris. The supernatant was collected and separated by ultracentrifugation (60 min, 250000 × g) into soluble (cytoplasmic and periplasmic) and total membrane fractions. Proteins in the soluble fractions were concentrated by chloroform-methanol precipitation [39], while the membrane fractions were washed with and resuspended in precipitation buffer. For differential extraction, triton X-100 was added to the membrane suspension to a final concentration of 1% (w/v), and the mixture was incubated on ice for 30 min and fractionated by ultracentrifugation (45 min, 230000 × g). Sucrose density gradients were prepared by layering solutions containing decreasing sucrose concentrations (60% (w/v) to 25% (w/v)) in triethanolamine buffer (50 mM triethanolamine, pH 7.5; 1 mM EDTA) upon one another in SW41 rotor tubes, followed by overnight equilibrium centrifugation at 4°C. Total membranes resuspended in triethanolamine buffer were layered on top of the gradients, and the tubes were centrifuged at 4°C (18 h, 270000 × g). Fractions were collected from the density gradients, and proteins were concentrated by precipitation as above.

Limited Proteinase K Digestion

For limited proteinase K digestion, bacteria were grown on agar plates for 24 hours, harvested and washed in PBS, and resuspended at a density of 6 × 10⁷ cells per ml in proteinase K buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM KC1) containing 1 mg/ml proteinase K (Sigma). This suspension was incubated at room temperature for 30 min. To stop the reaction, PMSF was added to a final concentration of 1 mM. Bacteria were collected by centrifugation at 5000 × g for 20 min at 4°C. Pellets were resuspended in radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-10, 0.25% sodium deoxycholate, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin) and mixed with SDS-PAGE sample solution.

Immunoprecipitation

Bacteria grown on agar plates were suspended in PBS and washed twice. An amount of 5 × 10¹⁰ bacteria was resuspended in radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-10, 0.25% sodium deoxycholate, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin) and the cells were lysed by sonication. Unbroken cells were removed by centrifugation for 10 min at 10000 × g. To remove unspecifically interacting proteins, the lysates were incubated with prewashed protein G-agarose (Roche Diagnostics) for 2h at 4°C, and then centrifuged. To the supernatants, 5 μl of the appropriate polyclonal antisera was added, and samples were incubated for 3h at 4°C. Then, 50 μl of prewashed protein G-agarose was added and samples were incubated at 4°C for additional 2h. After three washing steps with RIPA buffer, proteins were eluted with 100 mM glycine, pH 2.7, or by boiling in SDS-PAGE sample solution.

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Author Contributions

Conceived and designed the experiments: RH. Performed the experiments: KTP EW LF JS UB. Analyzed the data: KTP UB RH WF. Conceived the reagents/materials/analysis tools: LFJS WF. Wrote the paper: WF.
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